IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF

GHRELIN *O*-ACYLTRANSFERASE (GOAT)

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DEDICATION

This work is dedicated to my wife Wenwen Zeng, for her love and support

IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF GHRELIN *O*-ACYLTRANSFERASE (GOAT)

by

JING YANG

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GHRELIN *O*-ACYLTRANSFERASE (GOAT)

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Ghrelin is a 28-amino acid, appetite-stimulating hormone secreted by the food-deprived stomach. Ser-3 of ghrelin is acylated with an eight-carbon fatty acid, octanoate, which is critically required for its endocrine actions. However, the octanoylating enzyme had remained elusive for nearly a decade. By expression cloning, I have identified GOAT (Ghrelin *O*-Acyltransferase), an enzyme belonging to a family of 16 polytopic membrane-bound *O*-acyltransferases. GOAT activity requires catalytic Asn and His residues, which

are conserved through vertebrates. Consistent with its function, GOAT mRNA is largely restricted to stomach and intestine, the major ghrelin-secreting tissues. To further characterize GOAT function biochemically, I have developed a robust *in vitro* assay using membranes from insect cells infected with baculovirus encoding recombinant mouse GOAT. GOAT-containing membranes catalyze the transfer of [^{3}H]octanoyl from [^{3}H]octanoyl CoA to recombinant proghrelin *in vitro*. 50 μM palmitoyl CoA is necessary in the assays to prevent the deacylation of [^{3}H]octanoyl CoA by crude membrane preparations. Maximal GOAT activity is observed at pH 7.0, and there is no apparent requirement for metals as determined by a lack of inhibition by 1 mM EDTA. The apparent K_m for proghrelin is 6 μM and for [^{3}H]octanoyl CoA is 0.6 μM. The octanoylation reaction strictly depends on the GOAT recognition site comprising three of the four N-terminal amino acids of proghrelin: Gly-1, Ser-3, and Phe-4. A pentapeptide containing only the N-terminal five amino acids of ghrelin is octanoylated by the enzyme. Moreover, I have demonstrated that the activity of GOAT is subjected to end-product inhibition. Together, the insights provided by my research may facilitate the design of useful inhibitors of GOAT.

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PRIOR PUBLICATIONS

1. **Yang, J.**, Zhao, T-J., Goldstein, J.L., and Brown, M.S.: Inhibition of ghrelin *O*-acyltransferase (GOAT) by octanoylated pentapeptides. ***PNAS***, 105: 10750-10755, 2008.
2. **Yang, J.**, Brown, M.S., Liang, G., Grishin, N.V., and Goldstein, J.L.: Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. ***Cell***, 132: 387-396, 2008.
3. **Yang, J.**, Dong, C., Huang X., and Zhao, J.: Sulfonation of polyvinylidene difluoride resin and its application in extraction of restriction enzymes from DNA digestion solutions. ***Anal. Biochem.***, 322: 99-103, 2003.

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CHAPTER I: INTRODUCTION

Ghrelin as Natural Ligand for GHS-R

Ghrelin was named after the root word "*ghre*" in the Proto-Indo-European languages for "grow" (Kojima et al., 1999). Discovery of ghrelin can be traced back to the early 1980s, when studies showed that certain opioid derivatives and synthetic peptides induced growth hormone (GH) secretion from the pituitary (Bowers et al., 1980; Momany et al., 1981). Surprisingly, the synthetic compounds, or later known as growth hormone secretagogues (GHS), function independently of GH-releasing hormone receptor (GHRH-R), suggesting that GHS probably acts through an unidentified receptor. It took more than a decade before the receptor, namely the growth hormone secretagogue receptor (GHS-R), was identified in 1996 by expression cloning (Howard et al., 1996). Both GHS-R and GHRH-R are classic G-protein-coupled receptors, but they trigger GH release through distinct downstream events. Binding of GHS to GHS-R activates the phospholipase C pathway leading to release of Ca^{2+} from intracellular stores, whereas GHRH acts on GHRH-R to increase intracellular levels of cyclic AMP.

Whether GSH-R had a natural ligand, or whether the GH-releasing activity of the receptor was only a pharmacological effect, remained an open question for several years. Using intracellular free Ca^{2+} as the readout for GHS-R activation, Kojima et al. biochemically purified the endogenous ligand for GHS-R, unexpectedly from extracts of rat stomach (Kojima et al., 1999). The group named the peptide ligand "ghrelin" referring to its GH-releasing function. Ghrelin showed GH-releasing properties similar to the synthetic GHS, i.e., it acutely

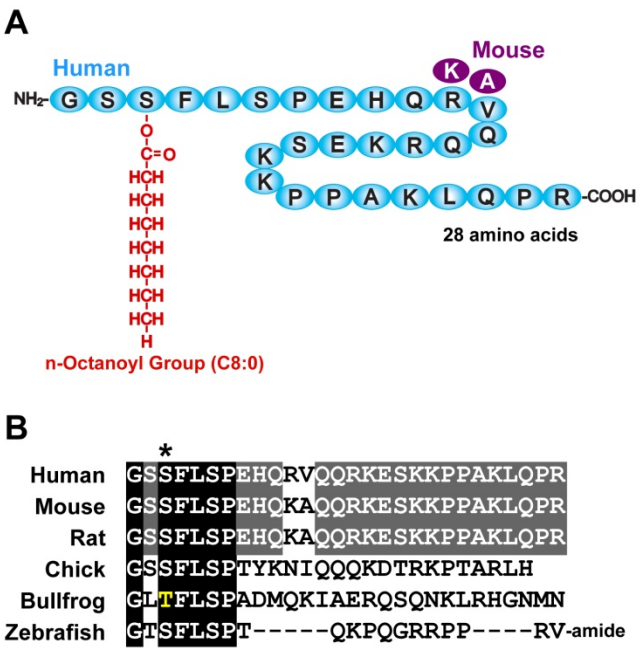

Figure 1. Amino Acid Sequence and Structure of Ghrelins
(A) Amino acid sequence of human ghrelin. The two residues different in mouse ghrelin are shown in purple. Octanoyl group linked to Ser-3 residue is colored in red. **(B)** Sequence alignment of ghrelins in vertebrates. Ghrelin homologs of human, mouse, rat, chick, bullfrog, and zebrafish are aligned. The residues conserved in all the species are boxed in black, whereas those conserved only in mammals are shaded in gray. Asterisk (*) denotes the residue position of ghrelins that is octanoylated.

stimulated GH secretion in both cultured pituitary cells and living rodents. The discovery of ghrelin provided definitive proof for the existence of a ghrelin-GHS / GHS-R signaling pathway in the regulation of GH secretion.

Structure and Post-Translational Processing of Ghrelin

Rat ghrelin is a 28-amino acid peptide (Kojima et al., 1999) (Fig. 1A). Due to alternative gene splicing, a second form of ghrelin (des-Gln14-ghrelin) exists as a 27-amino acid peptide with a sequence that is identical to ghrelin, except the Gln residue at position 14 is missing (Hosoda et al., 2000). The presence of ghrelin homologs has been demonstrated in different species of vertebrates, e.g., mouse, human, chick, bullfrog, and zebrafish. The amino acid sequence of ghrelin is highly conserved among mammals, i.e., it is identical in rat and mouse, and differs in human by only two residues. More strikingly, six of the seven

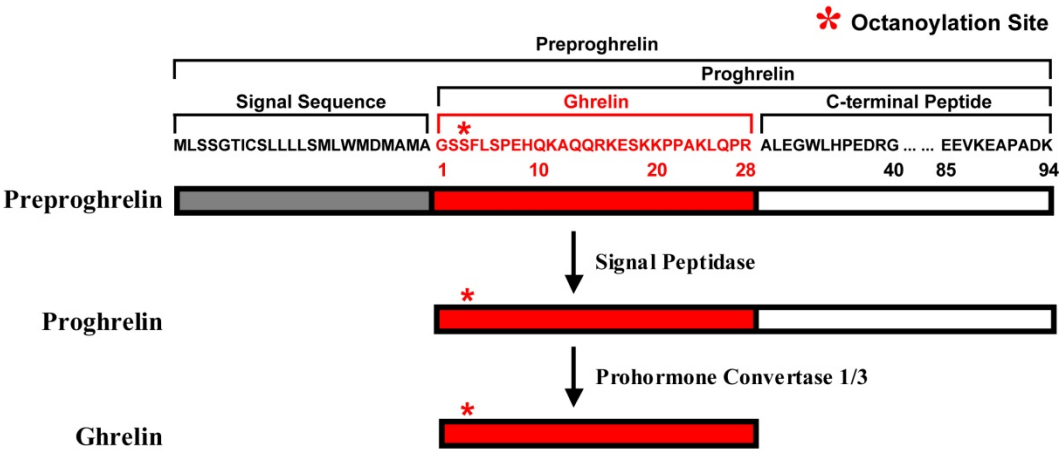

Figure 2. Proteolytic Processing of Preproghrelin to Ghrelin

Preproghrelin is processed to proghrelin by cleavage between Ala and Gly, which removes signal sequence. N-terminal Gly residue of proghrelin is hereafter referred as position 1 through the description. Proghrelin is further processed to ghrelin by prohormone convertase 1/3, which cleaves between Arg-28 and Ala-29 to release C-terminal peptide. Asterisk (*) denotes the octanoylated Ser-3 of ghrelin.

N-terminal amino acids of the peptide are absolutely conserved through vertebrates including mammal, bird, amphibian and fish (Fig. 1B). With the report that the synthetic peptides encompassing the N-terminal four or five residues of ghrelin could activate GHS-R as efficiently as full-length ghrelin (Bednarek et al., 2000), it is very likely that the N-terminal segment of ghrelin has been preserved over evolution as a core module for binding to GHS-R. Consistent with this notion, protein sequences of GHS-R are also highly conserved in vertebrates (Smith et al., 1999).

Like other peptide hormones, ghrelin is proteolytically derived from a precursor of 117 amino acids, preproghrelin (Fig. 2). The removal of the signal sequence generates proghrelin with 94 amino acids. In ghrelin-producing cells, prohormone covertase 1/3 (PC1/3) cleaves at the C-terminus of proghrelin to

release the 28 amino-acid ghrelin (Zhu et al., 2006). A recent study claimed that the C-terminal peptide of proghrelin could further give rise to another peptide hormone of 23 amino acids called obestatin (Zhang et al., 2005). However, the roles of obestatin as a ligand for the G-protein coupled receptor GPR39 or as a ghrelin antagonist have been refuted (Chartrel et al., 2007; Gourcerol et al., 2007a; Gourcerol et al., 2007b; Nogueiras et al., 2007). At present, there is no clear evidence for any function for the C-terminal peptide of proghrelin.

As a unique structural feature, the Ser-3 residue of rat ghrelin is modified by *O*-linked acylation with an eight-carbon fatty acid, octanoate (Kojima et al., 1999) (Fig. 1A). *O*-acylation on Ser-3 was subsequently demonstrated among ghrelin homologs in mammals, birds, and fish (Kojima and Kangawa, 2005). Even in the case of bullfrog ghrelin, where Ser-3 is replaced by Thr (Fig. 1B), the Thr residue is also octanoylated (Kaiya et al., 2001). Presently, ghrelin is the only known peptide (or protein) that is subject to *O*-acylation by medium-chain fatty acids.

Since the identification of the peptide, numerous studies have demonstrated that octanoylation of ghrelin is critically required for GH-releasing activity (Kojima et al., 1999) as well as other endocrine functions (see below). Thus, it is obviously important to reveal the octanoylating enzyme for a comprehensive understanding of the production of ghrelin and its physiology. However, the enzyme(s) that catalyzes this reaction has remained elusive despite extensive searches by many laboratories.

Orexigenic Effect and Metabolic Functions of Ghrelin

Discovered as a GH-releasing peptide, what really brought ghrelin to the

limelight in metabolism was its abilities to increase appetite and body weight (Tschop et al., 2000). Historically, people have realized that apart from GH-releasing activity, GHS could cause a short-lived surge in food intake when administered either peripherally or centrally (Locke et al., 1995; Torsello et al., 1998). Thus, as a natural ligand for GHS-R, it appeared logical that ghrelin might exert an effect on appetite regulation. Researchers have shown that central or peripheral infusion of ghrelin in rodents markedly enhanced food intake (Nakazato et al., 2001; Wren et al., 2001b), and intriguingly, the appetite-stimulating effect largely depended on the presence of the octanoyl group on the peptide (Toshinai et al., 2006). A similar orexigenic effect of ghrelin was also observed in humans upon peripheral administration (Wren et al., 2001a). In addition, the endogenous concentration of ghrelin in human plasma rose immediately before each meal, and decreased sharply post-prandially (Cummings et al., 2001; Liu et al., 2008), further suggesting an association of ghrelin with hunger and appetite. Until now, ghrelin has been the only known orexigenic hormone circulating in humans.

Mainly produced in the stomach, an ideal organ to sense the energy intake and food volume in humans, ghrelin has emerged as an important regulator in growth and metabolism. Ghrelin is proposed to exert the effects on appetite and body weight through a central action in the hypothalamus (Cummings, 2006; Kojima and Kangawa, 2006). GHS-R mRNA is highly expressed in the arcuate nucleus (ARC) of the hypothalamus, and the binding of ghrelin to the receptor causes an increased expression of neuropeptide Y (NPY) and agouti-related protein (AGRP) (Nakazato et al., 2001), which two molecules are well known as signals to

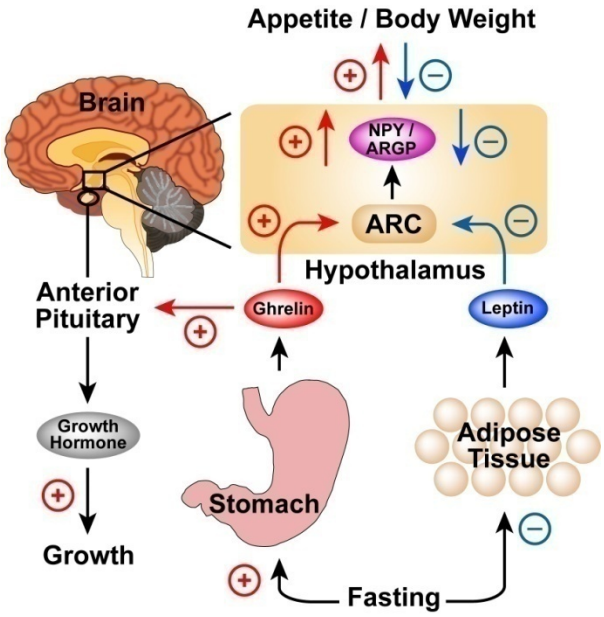

Figure 3. Central Actions of Ghrelin Secreted from the stomach, ghrelin acts on anterior pituitary to induce GH secretion, resulting in the growth of human bodies. At the same time, binding of ghrelin to GHS-R in ARC neurons of the hypothalamus stimulates the expression of NPY/ARGP, which increase appetite and body weight. As a balancing mechanism for ghrelin, leptin released by adipocytes acts on ARC neurons to decrease the expression of NPY/ARGP, leading to a reduction of food intake and weight gain. Fasting suppresses leptin but increases ghrelin secretion, resulting in an overall activation of orexigenic pathway.

stimulate food intake and weight gain (Coll et al., 2007). Therefore, when coupled with its GH-releasing activity on the anterior pituitary, ghrelin could induce an anabolic state in human bodies (Fig. 3).

Owing to the weak blood-brain barrier in the region, ARC neurons are positioned to be in direct contact with peripheral signals, such as the peptide hormone leptin, which is a 16-kDa peptide secreted by adipocytes in proportion to body fat and is a crucial element in regulating body weight (Coll et al., 2007). Acting in an opposite way to ghrelin, binding of leptin to its receptor in ARC neurons causes a decreased level of NPY / AGRP, resulting in a reduction of food intake and body weight. In fact, the anorexigenic effects of leptin were readily reversed by the simultaneous administration of ghrelin (Nakazato et al., 2001), indicating that ghrelin and leptin probably share a common neuronal pathway in the hypothalamus. In awareness of the counter-acting functions of ghrelin and

leptin, people have proposed that the two peripheral hormones evolve together to balance with each other in maintaining an appropriate energy intake and a constant body weight (Fig. 3) (Kojima and Kangawa, 2006).

Given the well-documented orexigenic effect of ghrelin, reports of mice with gene-targeted deletions of ghrelin exhibiting no changes in feeding behavior or body composition were surprising (Sun et al., 2003; Wortley et al., 2004). However, more detailed studies revealed that the elimination of ghrelin or GHS-R in mice caused a modest but significant reduction in weight gain when the animals were presented with high fat diets (Wortley et al., 2005; Zigman et al., 2005). The latter data support the notion that ghrelin functions as an important component in the control of energy homeostasis, particularly under conditions of energy overload similar to diet-induced obesity in humans.

Recently, in the broader scenario of metabolism, additional functions of ghrelin have started to be unveiled. Studies showed ghrelin could enhance glucose-stimulated insulin secretion and improve peripheral insulin sensitivity in rodents (Gauna et al., 2004; Heijboer et al., 2006; Yada et al., 2008), and an association between ghrelin and insulin resistance has been suggested in humans (Barazzoni et al., 2007; McLaughlin et al., 2004; Poykko et al., 2003). However, contradictory conclusions exist among independent reports, and it has not been clearly demonstrated that the absence of ghrelin signaling can improve insulin secretion or peripheral insulin sensitivity in mice (Pfluger et al., 2008). Thus, a definitive role of ghrelin in glucose metabolism and insulin sensitivity still remains under debate, even though it does not diminish the possibility of a metabolic association of ghrelin with insulin action. Evidence has also started to

accumulate that central administration of ghrelin might regulate adipocyte metabolism by increasing triglyceride uptake and lipogenesis, while inhibiting lipid oxidation in white adipocytes (Theander-Carrillo et al., 2006). Taken together, the metabolic functions of ghrelin distinct from appetite regulation should be considered during any pharmacological manipulation of the peptide in animals and humans.

Ghrelin Pathway as Target to Treat Obesity

Obesity is a morbid condition that not only limits patient activities, but also is a major risk factor for other life-threatening pathologies such as cardiovascular disease, non-insulin-dependent diabetes mellitus (type-2 diabetes), and certain cancers. According to the World Health Organization, 300 million people are clinically diagnosed as obese worldwide, and obesity is rapidly evolving into a serious health issue, especially in industrialized nations. Given a global estimation of 10,000 premature deaths per week due to obesity, an effective anti-obesity drug could save many lives.

One of the current efforts in the search for anti-obesity therapies has been the identification of appetite-suppressing agents. The basic assumption behind this quest is simple, i.e., people will eat less if they do not feel hungry. Given numerous studies on the orexigenic effect of ghrelin, in particular, the finding that ghrelin levels in human plasma show a strong correlation with hunger (Liu et al., 2008), it is not surprising that considerable interest has arisen in ghrelin inhibitors as potential appetite-suppressing drugs.

Several pharmacological approaches to intervene in ghrelin signaling have

been proposed and investigated by both researchers and for-profit enterprises. One approach is to ablate the presence of ghrelin in plasma, either by inhibition of ghrelin secretion (Perez-Tilve et al., 2007), or by neutralization of circulating ghrelin with specific antibodies (Zorrilla et al., 2006). Another approach has been to manipulate GHS-R through occupation of the receptor by synthetic ligands of various effects. Since most known GHS-R ligands are agonists, e.g., GHS, the current search has focused on developing the antagonists and inverse agonists. The rationale is that antagonists may acutely block pre-prandial ghrelin action to cause people to eat less at each meal, whereas inverse agonists might be useful to reduce the constitutive buildup of hunger between meals.

Since octanoylation is crucial for ghrelin endocrine functions, a novel approach to antagonize ghrelin would be to inhibit the enzyme that octanoylates the peptide. Such an inhibitor should be quite specific since no other proteins are known to be octanoylated in humans, which may help avoiding side-effects. Additionally, inhibition of the octanoylating enzyme should not affect the production of desacyl-ghrelin (ghrelin without the octanoyl group), which could minimize an interference with any function associated with the unmodified peptide. Moreover, small-molecule inhibitors for an enzyme are generally easier to obtain than mimetics of peptide hormones. Therefore, inhibitors for the octanoylating enzyme appear as an elegant choice for anti-obesity therapies in ghrelin pathway.

Fatty Acylation and Protein Acyltransferases

Protein modification with long-chain fatty acids is a universal phenomenon in

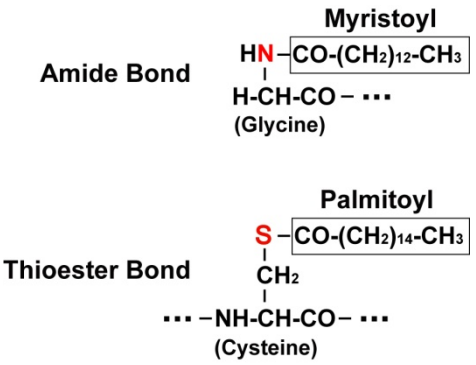

Figure 4. Two Classes of Fatty Acylation in Eukaryotic Cells
N-myristoylation occurs on N-terminal glycine residue via amide linkage, whereas *S*-palmitoylation on cysteine residue through thioester bond.

eukaryotic cells. Based on differences in temporal and chemical features, fatty acylation has been historically divided into two classes, i.e., *N*-myristoylation (amide linkage of 14-carbon myristate) and *S*-palmitoylation (thioester linkage of 16-carbon palmitate) (Magee and Courtneidge, 1985; Olson and Spizz, 1986; Olson et al., 1985) (Fig. 4). *N*-myristoylation occurs co-translationally on the N-terminal glycine residue of cytosolic proteins, and this chemical linkage is thought to be irreversible and highly resistant to hydroxylamine treatment (Johnson et al., 1994). On the contrary, protein *S*-palmitoylation, which happens post-translationally to cysteine residue, is generally regarded as reversible and sensitive to deacylation by hydroxylamine (Smotrys and Linder, 2004). With accumulating evidences of diverse fatty acylation, this categorization has been expanded to include attachment of fatty acids to other amino acids in either amide- or ester-linkage. For instance, palmitoylation on N-terminal cysteine residue through an amide linkage has been described for Sonic Hedgehog (Pepinsky et al., 1998). Also, the attachment of a monounsaturated fatty acid to serine through an ester linkage has been reported for Wnt (Takada et al., 2006).

Currently, three different classes of protein acylating enzymes are known in eukaryotic cells. *N*-myristoylation is catalyzed by *N*-myristoyl transferase (NMT),

which enzymology has been firstly documented (Johnson et al., 1994). Two isozymes of NMT exist in mammalian genomes, i.e., NMT1 and NMT2, both of which myristoylate cytosolic proteins like c-Src, ARF and G_{α}. The second class of acylating enzymes are palmitoyl *S*-transferases, which share a common DHHC (Asp-His-His-Cys) catalytic motif (Lobo et al., 2002; Mitchell et al., 2006). 23 members of DHHC family are predicted in the mouse genome (Fukata et al., 2004). Similar to NMTs, all characterized members of DHHC family modify only cytosolic proteins, e.g., Ras, RhoB and PSD-95. The third class of protein acylating enzymes belong to the family of membrane-bound *O*-acyltransferases (MBOAT) (Hofmann, 2000). There are two unique features of MBOAT family, first, substrates of family members are sheerly diverse and include sterols, phospholipids and proteins (Fig. 16). Second, for the two MBOATs known to attach fatty acyl groups to proteins, hedgehog acyltransferase (HHAT) and porcupine (PORC), the substrates are the *secretory* proteins Sonic Hedgehog and Wnt, respectively (Chamoun et al., 2001; Willert et al., 2003). However, prior to the current work, there were no reports of protein acyltransferases specific for medium-chain fatty acids such as octanoate.

CHAPTER II: PART I: Identification of Ghrelin *O*-Acyltransferase

Proteolytic Processing of Preproghrelin in Endocrine Cell Lines

As the first step in identifying ghrelin-octanoylating enzyme, I screened for cancer cell lines that could proteolytically process preproghrelin to ghrelin. The establishment of a ghrelin-producing cell system turned out to be crucial, since it later enabled me to carry out expression cloning in pursuit of an elusive octanoylating enzyme.

For this purpose, I expressed preproghrelin in more than 50 different cancer cell lines (Table 1) through cDNA transient transfection. Peptide extracts of transfected cells were subjected to 16% Tricine SDS-PAGE and immunoblotted with a polyclonal antibody that recognized both desacyl- and octanoyl-ghrelin (94C, see **Experimental Procedures**). All of the transfected cells produced a peptide with an apparent molecular mass of 12 kDa, corresponding to proghrelin

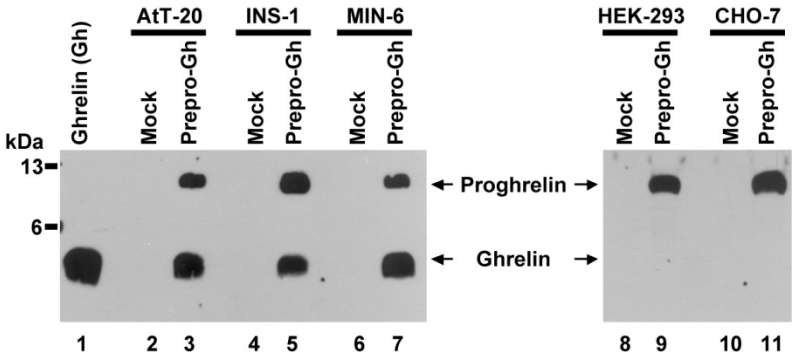

Figure 5. Proteolytic Processing of Preproghrelin in Endocrine Cell Lines

The indicated cells were set up for experiments on day 0. On day 2, one dish of each cell line was transfected with 5 μg of mouse preproghrelin cDNA, and a second dish was subjected to mock transfection with pcDNA3.1. On day 4, cells were harvested, and peptides were extracted for SDS-PAGE and immunoblot analysis. Lane 1 contains synthetic octanoyl-ghrelin.

with the signal sequence removed (Fig. 5, data not shown). Particularly, in three endocrine cell lines, mouse pituitary tumor AtT-20, rat insulinoma INS-1, and mouse insulinoma MIN-6, a smaller peptide with an apparent molecular mass of 3 kDa was detectable (Fig. 5, left panel), suggesting that these three cell lines could proteolytically process proghrelin to ghrelin. The processing of proghrelin was not observed in non-endocrine cell lines, e.g., human kidney HEK-293 and Chinese hamster ovary CHO-7 (Fig. 5, right panel).

To confirm that the 3-kDa band detected in INS-1 cells resulted from cleavage between Arg28 and Ala 29 of proghrelin, I prepared cDNAs encoding mutant

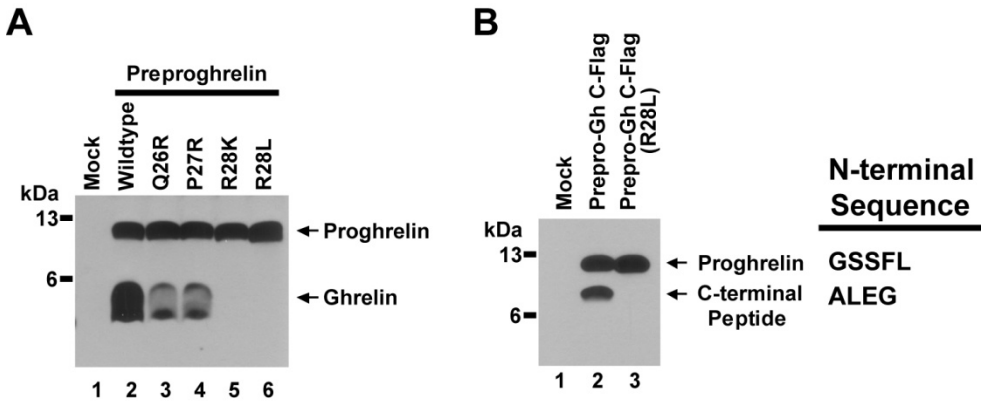

Figure 6. Cleavage Sites of Preproghrelin in INS-1 cells

(A) Effect of cleavage-site mutations on the processing of proghrelin. INS-1 cells were set up on day 0, and transfected on day 2 with 6 μg indicated wild-type or mutant versions of preproghrelin cDNA. On day 4, the peptides were extracted for SDS-PAGE and immunoblot analysis.

(B) N-terminal sequencing of proghrelin and C-terminal peptide. On day 0, 30 dishes of INS-1 cells were set up for experiments. On day 2, the cells were transfected with 5 μg of a cDNA encoding mouse preproghrelin with a C-terminal Flag-tag. On day 4, the cells were solubilized in PBS containing Triton X-100, and a small aliquot of 100,000*g* supernatant was subjected to SDS-PAGE and immunoblotted with anti-Flag antibody (*left panel*). The remainder of 100,000*g* supernatant was treated with anti-Flag affinity beads, after which the eluted proteins were subjected to SDS-PAGE. N-terminus of the separated peptides corresponding to proghrelin and its C-terminal peptide were sequenced as described in **Experimental Procedures** (*right panel*).

versions of preproghrelin with amino-acid substitutions at or near Arg28. Replacement of Arg28 with either Lys or Leu abolished cleavage, whereas replacement of residue 26 or 27 with Arg reduced cleavage (Fig. 6A). To further define the cleavage sites on preproghrelin, INS-1 cells were transfected with cDNA encoding preproghrelin containing a C-terminal Flag-tag, and Flag-tagged peptides were isolated by adherence to immune-affinity beads. Purified proghrelin and its C-terminal peptide were separated on SDS-PAGE, and subjected to protein sequencing (Fig. 6B). The N-terminal sequence of Flag-tagged proghrelin was GSSFL, consistent with authentic proghrelin after removal of the signal sequence. The N-terminal sequence of the C-terminal peptide was ALEG, consistent with cleavage after Arg28 to generate ghrelin. Considered together, INS-1 and the other two endocrine cells were able to process preproghrelin at the correct sites to produce authentic ghrelin.

Next, I set out to address the question of whether ghrelin produced by any of endocrine cell lines could be octanoylated. A reverse-phase chromatographic assay was developed to efficiently separate desacyl-ghrelin from octanoyl-ghrelin. For use as standards, synthetic desacyl- and octanoyl-ghrelin peptides were obtained. The peptides were applied to a C18 reverse-phase column and eluted with a step-gradient of 20%-, 40%-, and 80%-CH_3CN in 0.1% TFA (see **Experimental Procedures**). Eluted peptides in the three fractions were examined by immunoblot analysis. As shown in the upper panel of Fig. 7, desacyl-ghrelin was eluted exclusively in 20%-CH_3CN fraction, whereas octanoyl-ghrelin was present in 40%-CH_3CN fraction due to ther hydrophobic octanoyl group. When ghrelin peptides extracted from transfected endocrine cell lines were fractionated

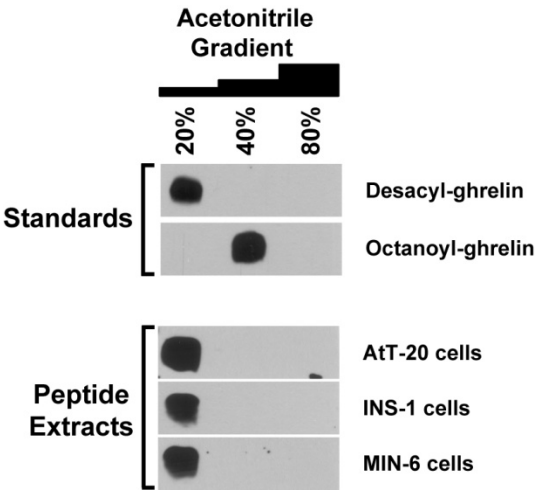

Figure 7. Production of Desacyl-Ghrelin in Endocrine Cell Lines
The indicated cells were set up for experiments on day 0. On day 2, cells were transfected with 3 μg mouse preproghrelin cDNA and 2 μg pcDNA3.1 mock vector. On day 3, each dish of cells received a direct addition of 100 μM octanoate-albumin. On day 4, four dishes of each cell line were harvested and pooled, and the extracted peptides were fractionated by reverse-phase chromatography as described in **Experimental Procedures**. Only the protein corresponding in molecular mass to ghrelin is shown.

by reverse-phase chromatography, all of the ghrelin signal was detected in 20%-CH_3CN fraction, indicating that none of the three endocrine cell lines could octanoylate ghrelin (Fig. 7, lower panel). The simple explanation to this negative result was that the octanoylating enzyme(s) was missing in the cells. Therefore, I decided to pursue an expression cloning strategy for the enzyme. All further experiments were carried out in INS-1 cell line, which was easiest to maintain and transfect.

16 Membrane-Bound *O*-Acyltransferases in Mouse Genome

For a candidate approach to expression cloning, we had been focusing on members of membrane-bound *O*-acyltransferase (MBOAT) family. All of these enzymes are postulated to transfer fatty acyl groups to hydroxyl or sulfhydryl groups, forming ester or thioester bonds. Among the known substrates are lipids such as cholesterol and diacylglycerol. Two members of the MBOAT family,

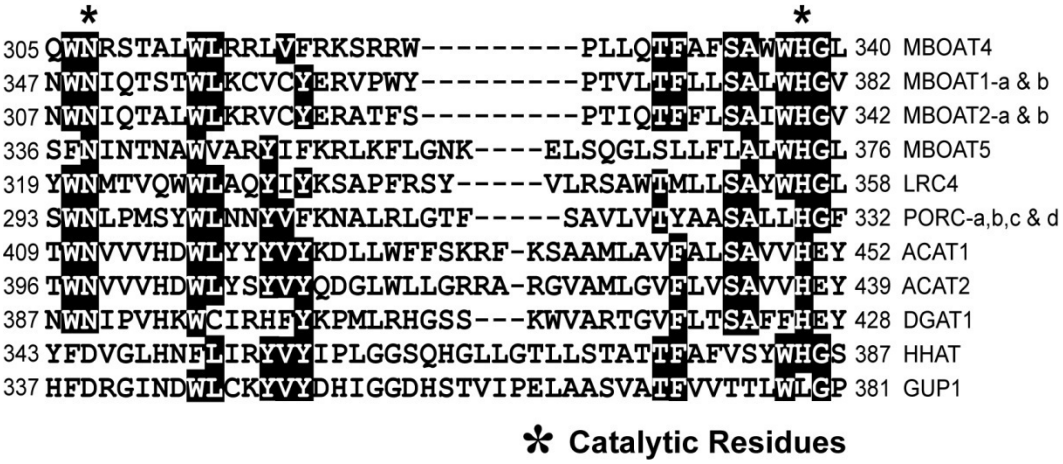

Figure 8. Alignment of Conserved Region of 16 MBOATs in Mouse Genome

Amino acid residues conserved in more than 50% of the sequences are shaded. Asterisks (*) denote the putative catalytic Asn and His residues. In the case of MBOATs with different isoforms (MBOAT1, MBOAT2, and PORC), for simplicity, the numbering refers to isoform-a.

HHAT and PORC, have been shown to acylate the secretory proteins Sonic Hedgehog and Wnt, respectively (Chamoun et al., 2001; Willert et al., 2003). Particularly, we also became aware that PORC could acylate a serine residue in Wnt (Takada et al., 2006). Since the octanoylating enzyme also attaches an acyl group to a serine residue of ghrelin, it was logical to hypothesize that the enzyme could be one member of the MBOAT family.

In collaboration with Dr. Nick V. Grishin (University of Texas Southwestern Medical Center), 16 different MBOATs were identified in mouse genome by bioinformatic analyses. The criteria for inclusion were based on the original work of Hofmann (Hofmann, 2000), who initially defined this family. Fig. 8 showed the alignment of conserved sequences of putative catalytic domains in mouse MBOAT proteins. These 11 catalytic domains were found in 16 MBOAT proteins, since two of the encoding genes give rise to two isoforms and one gives rise to

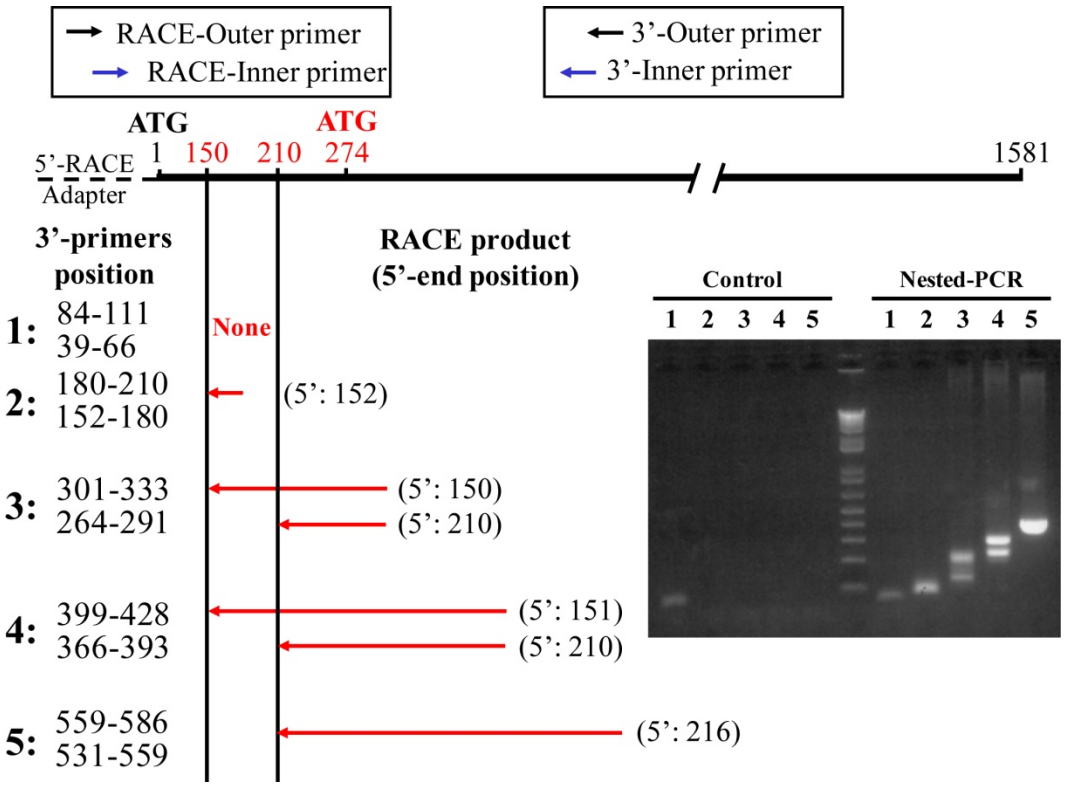

Figure 9. 5'-RACE of MBOAT4 Transcript

Diagram summarizes the result of 5'-RACE with five sets of 3'-nested primers. 1st set of primers failed to generate specific PCR product. 2nd and 5th sets of primers individually gave rise to one PCR product, whereas 3rd and 4th sets individually resulted in two PCR products. Control samples were set up without the addition of stomach total RNA.

four isoforms with alternative splicing. Asn and His residues that were predicted to participate in catalysis were indicated.

Cloning of Ghrelin *O*-Acyltransferase

Full-length cDNAs encoding 15 MBOATs were successfully cloned from mouse stomach RNA. However, I was initially unable to clone a full-length cDNA for the 16th MBOAT by RT-PCR. This MBOAT was designated in NCBI database (May 2007) as ‘‘similar to *O*-acyltransferase (membrane bound) domain

containing 1'' (XM_134120). In retrospect, efforts to clone its cDNA failed because NCBI annotation at the 5'-end of mRNA was incorrect, and as a result, the 5'-primers were not able to prime PCR amplification. The project was slipping into in an inevitable failure until the difficulty was overcome through an unusual approach, i.e., after obtaining four DNA segments corresponding to nucleotides 1-391, 398-885, 907-1254, and 1261-1581 of XM_134120 (synthesized by Integrated DNA Technologies, Coralville, IA), I pieced them together by fusion-PCR (Karreman, 1998) to generate an artificially synthetic cDNA corresponding to the full-length sequence of XM_134120.

In pilot experiments in INS-1 cells, transfection of the synthetic cDNA was observed to produce ghrelin-octanoylating activity (data not shown). On June 20, 2007, the incorrect NCBI annotation of XM_134120 was replaced by two new annotations that were renamed MBOAT4, XM_001476434 and XM_001472220. These two versions of MBOAT4 differed from each other by 376 nucleotides at the 5'-end, and they differed from XM_134120 at the 5'-end in the following ways: XM_001476434 was 211 bp shorter than XM_134120, and XM_001472220 was 165 bp longer than XM_134120. To determine the correct 5'-end of MBOAT4 mRNA, 5'-rapid amplification of cDNA ends (5'-RACE) was carried out using total RNA from mouse stomach, five different sets of 3'-nested primers designed according to the original annotated transcript XM_134120 (Table 2), and FirstChoice RLM-RACE Kit (Ambion). Based on the results of 5'-RACE (Fig. 9), XM_001476434 was shown to be the correct annotation MBOAT4 mRNA, which was subsequently proven to encode authentic Ghrelin *O*-Acyltransferase (GOAT).

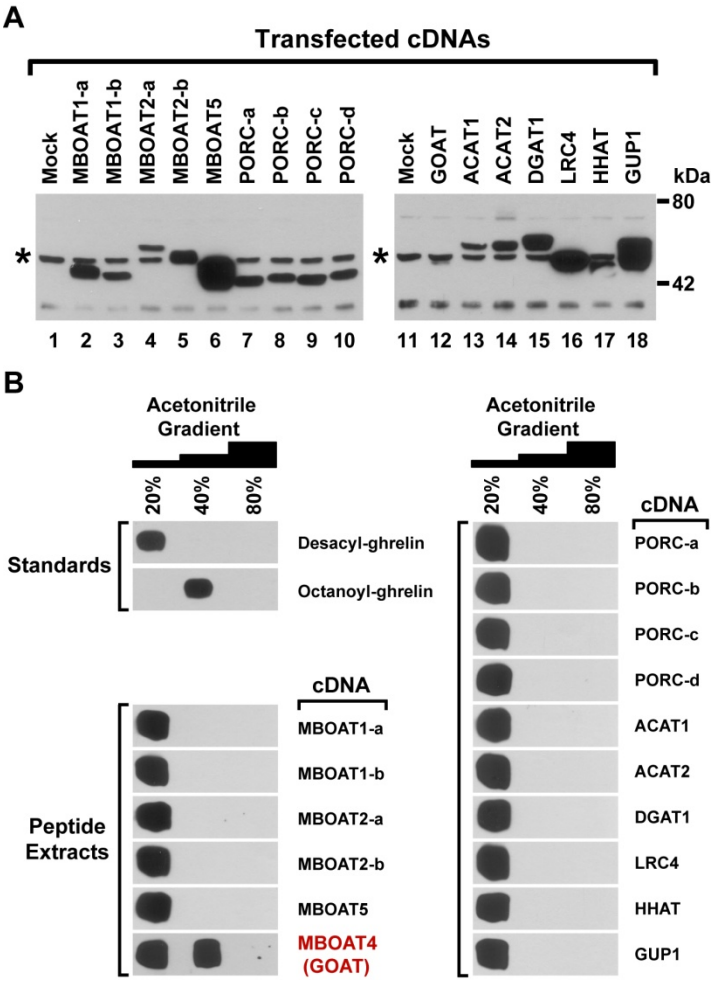

Figure 10. Identification of GOAT by Expression Cloning in INS-1 Cells
INS-1 cells were set up on day 0. **(A)** Expression of 16 MBOATs in INS-1 cells. On day 2, cells were transfected with 5 μg of cDNA encoding indicated MBOATs with a C-terminal Flag-tag. On day 4, membrane fractions were prepared and subjected to immunoblot analysis with anti-Flag antibody. Asterisk (*) denotes an irrelevant cross-reacting band present in membrane fraction of INS-1 cells.
(B) Identification of GOAT. On day 2, cells were transfected with 5 μg of preproghrelin and 0.2 μg of cDNA encoding indicated MBOAT. On day 5, the peptides were extracted and subjected to reverse-phase chromatography.

Fig. 10 shows a series of experiments designed to determine whether any of 16 MBOATs were capable of producing octanoyl-ghrelin when co-expressed with preproghrelin in INS-1 cells. I first prepared cDNAs encoding each of the MBOATs with C-terminal Flag-tag. When transfected into INS-1 cells, all of the cDNAs produced individual MBOAT protein that could be detected by immunoblot analysis (Fig. 10A). The same set of cDNAs were then transfected

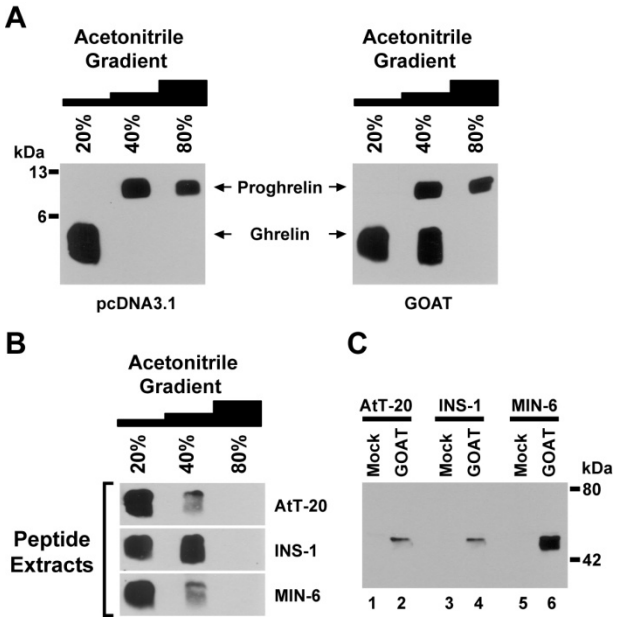

Figure 11. Octanoylation of Ghrelin in Endocrine Cell Lines

(A) Octanoylation of ghrelin by GOAT. On day 2, INS-1 cells were transfected with 5 μg of preproghrelin and 0.2 μg of mock vector pcDNA3.1 or GOAT. On day 5, the peptides were extracted and subjected to reverse-phase chromatography. The bands corresponding to both proghrelin and ghrelin are shown.

(B) GOAT acylates ghrelin in three endocrine cell lines. The indicated cells were transfected and processed as described in (A), except only ghrelin band is shown.

(C) Expression of GOAT in endocrine cell lines. On day 2, the indicated cells were transfected with 6 μg GOAT with C-terminal HA-tag. On day 4, membrane fractions were prepared and subjected to immunoblot analysis with anti-HA antibody.

into INS-1 cells together with preproghrelin. Ghrelin peptides were extracted and subjected to reverse-phase chromatography. MBOAT4 (hereafter designated as GOAT) was the only MBOAT that produced acylated ghrelin, which appeared as a 3-kDa band in 40%-CH_3CN fraction (Fig. 10B).

To confirm the octanoylating activity of GOAT, co-transfection experiment was repeated with a control of mock vector (Fig. 11A). When preproghrelin cDNA was transfected together with pcDNA3.1, ghrelin emerged only in 20%-CH_3CN fraction, indicating the lack of octanoylation. Proghrelin emerged in 40%- and 80%-CH_3CN fractions even though it was presumably not acylated. The elution pattern of proghrelin could be explained by the tendency of longer peptides to adhere strongly to reverse-phase resins. When GOAT cDNA was included in the transfection, approximately 50% of ghrelin signal was shifted into

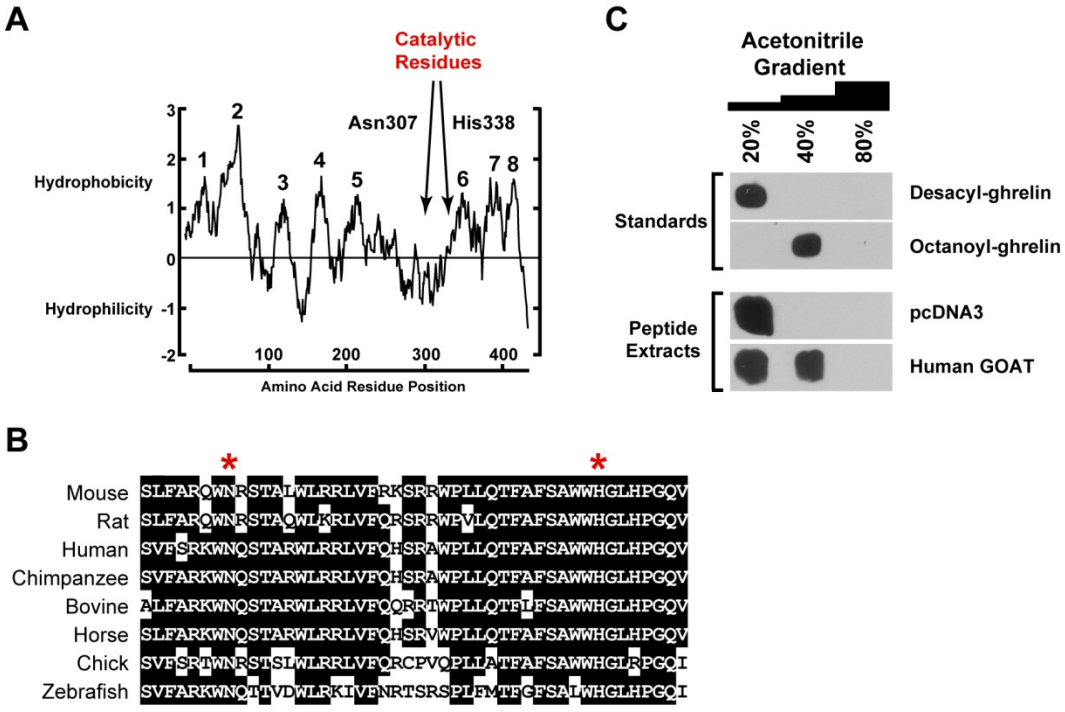

Figure 12. GOAT Orthologs in Vertebrate Species

(A) Hydropathy plot of mouse GOAT. Hydropathy index was calculated over a window of 18 residues, and the predicted eight transmembrane helices are numbered. Positions of the putative catalytic Asn and His residues are indicated.

(B) Alignment of the catalytic domain of GOAT orthologs. Amino acid residues conserved in more than 50% of the sequences are shaded. Asterisks (*) denote the catalytic Asn and His residues.

(C) Conserved activity of human GOAT. On day 2, INS-1 cells were transfected with 5 µg human preproghrelin and 0.2 µg mock vector pcDNA3.1 or human GOAT. On day 5, the peptides were extracted and subjected to reverse-phase chromatography.

40%-CH_3CN fraction. Of note, the elution pattern of proghrelin remained the same as in the control transfection. It was also observed that the octanoylating activity of GOAT was not restricted to INS-1 cells. Expression of GOAT produced octanoyl-ghrelin in each of three endocrine cell lines that were capable of processing proghrelin to ghrelin (Fig. 11B and C).

Fig. 12A showed a hydropathy plot of mouse GOAT sequence. GOAT was

suggested to contain eight transmembrane segments, a finding in keeping with other MBOATs, all of which have multiple membrane-spanning helices. GOAT sequence was highly conserved in mammalian and avian species, and a close ortholog was also identified in zebrafish (Fig. 12B). The putative catalytic Arg and His residues are conserved throughout. Moreover, the enzymatic activity of GOAT was conserved through vertebrates, for instance, human ortholog of GOAT efficiently octanoylated human ghrelin in INS-1 cells transfected with both cDNAs (Fig. 12C). In fact, the authenticity of human GOAT had been confirmed independently by another group (Gutierrez et al., 2008), which also surprisingly showed that human ghrelin could be a substrate for zebrafish GOAT.

Molecular Characterization of Octanoylating Activity of GOAT

To confirm that GOAT indeed modified ghrelin through *O*-acylation, I tested the lability of the modification to hydroxylamine treatment (see **Experimental Procedures**), which is known to release ester-bound fatty acids from proteins at alkaline pH (Bizzozero, 1995). As shown in the upper panel of Fig. 13A, when synthetic octanoyl-ghrelin was treated with 1 M hydroxylamine (pH 8.0), the peptide got de-acylated and no longer eluted from reverse-phase chromatography in 40%-CH_3CN fraction. As a control, treatment with 1 M Tris-Cl (pH 8.0) had no such an effect. The lower panel of Fig. 13A showed the result of hydroxylamine treatment of octanoyl-ghrelin partially purified from transfected INS-1 cells. When treated with 1M Tris-Cl, ghrelin was still eluted from reverse-phase chromatography in 40%-CH_3CN fraction. But when treated with 1 M hydroxylamine (pH 8.0), most of ghrelin peptide was reverted to 20%-CH_3CN

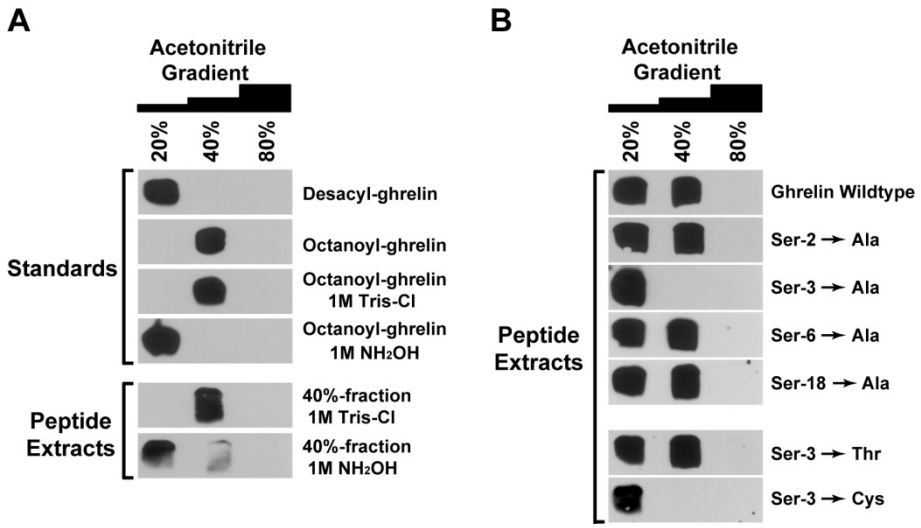

Figure 13. GOAT Modifies Ghrelin through *O*-linked Acylation on Ser-3 Residue

(A) Hydroxylamine treatment. On day 2, INS-1 cells were transfected with 5 μg mouse preproghrelin and 0.2 μg GOAT. On day 5, 10 dishes of cells were harvested and pooled, after which peptide extracts were processed for reverse-phase chromatography. The 40%-CH_3CN fraction and synthetic octanoyl-ghrelin standard were subjected to treatment with either 1 M Tris-Cl (pH 8.0) or 1 M NH_2OH (pH 8.0), followed by reverse-phase chromatography and immunoblot analysis.

(B) Mutational analysis. On day 2, INS-1 cells was transfected with 0.2 μg GOAT cDNA and 5 μg of cDNA encoding wild-type or indicated mutant versions of preproghrelin. On day 5, the peptides were extracted and subjected to reverse-phase chromatography.

fraction, indicating that the peptide had been de-acylated.

Octanoylation of ghrelin *in vivo* is known to occur at Ser-3 of the peptide. To test whether GOAT attached octanoyl group to the correct residue of ghrelin, mutagenesis analysis was carried out. Mutation of Ser-3 to Ala totally abolished the octanoylation by GOAT, whereas substitution of Ala for other serine residues in ghrelin (residues 2, 6, and 18) did not affect the modification (Fig. 13B), supporting that GOAT octanoylated the physiologic Ser-3 residue. Interestingly, replacement of Ser-3 with Thr preserved the octanoylation, a finding consistent

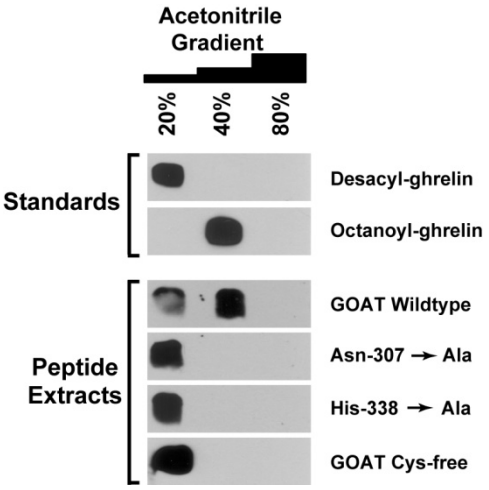

Figure 14. Identification of Residues in GOAT Required for Catalytic Activity

INS-1 cells were set up for experiments on day 0. On day 2, cells were transfected with 5 μg preproghrelin and 0.2 μg of cDNA encoding wild-type or indicated mutant versions of GOAT. On day 5, the peptides were extracted and subjected to reverse-phase chromatography.

with the observation that this position is occupied by an octanoylated threonine in bullfrog ghrelin (Kaiya et al., 2001). In contrast, ghrelin harboring substitution of Cys at Ser-3 failed to be modified by GOAT, suggesting that GOAT could only attach octanoyl group to form ester but not thioester bond.

As shown in Fig. 12A, the predicted catalytic residues in mouse GOAT were Asn-307 and His-338. Both of the two residues were required for the enzymatic activity of GOAT, since substitution of either residue with Ala abolished the ability of GOAT to acylate ghrelin (Fig. 14). Previous studies with another member of MBOAT family, ACAT1, have suggested there is likely no involvement of an thioester-intermediate with acyl group directly linked to the enzyme in acylation reaction, since Cys-free ACAT1 is still catalytically active (Lu et al., 2002). In contrast, when all of 14 cysteine residues in GOAT were mutated to Ala (Cys-free mutant), the mutant enzyme became totally inactive in transfected INS-1 cells (Fig. 14). However, it could not currently be ruled out that catalytic inactivation of Cys-free GOAT might be due to protein misfolding. Therefore, it remained an open question of whether a thioester-intermediate was

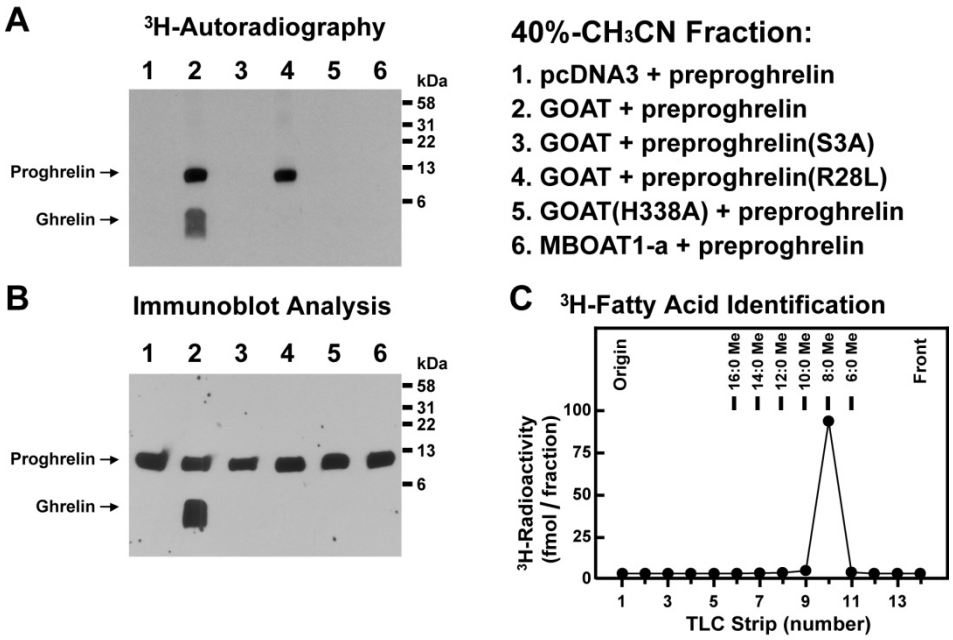

Figure 15. GOAT-Mediated Incorporation of [^{3}H]Octanoate into Proghrelin and Ghrelin (A and B) INS-1 cells were set up for experiments on day 0. On day 2, cells were transfected with 5 μg preproghrelin (wild-type or mutant) and 0.2 μg either GOAT (wild-type or H338A-mutant) or MBOAT1-a as indicated in Panels 1-6. On day 4, cells were incubated with [^{3}H]octanoate as described in **Experimental Procedures**. Each 40%-CH_3CN fraction of reverse-phase chromatography was subjected to either ^{3}H-autoradiography (A) or immunoblot analysis (B). (C) Identification of ^{3}H-labeled fatty acid on proghrelin and ghrelin. The identity of ^{3}H-fatty acid covalently attached to proghrelin and ghrelin in (A) was identified by fatty acid methyl ester analysis as described in **Experimental Procedures**. The positions of migration of FAME standards on reverse-phase TLC (C6:0 to C16:0 methyl esters) are shown at the top.

involved in GOAT-catalyzed octanoylation reaction.

To confirm that GOAT modified ghrelin with eight-carbon octanoate, I performed metabolic labeling experiments with [^{3}H]octanoate (see **Experimental Procedures**). INS-1 cells were transfected with cDNAs encoding preproghrelin together with wild-type or mutant version of GOAT, and incubated with [^{3}H]octanoate. Peptide extracts were fractionated on reverse-phase chromatography, and proteins in each 40%-CH_3CN fraction were subjected to

^{3}H-autoradiography. When the cells were transfected with GOAT cDNA, labeled peptides were observed in the positions of proghrelin and ghrelin (Fig. 15A, Lane 2). As expected, no radioactivity was incorporated into S3A-mutant of ghrelin (Lane 3). Lane 4 showed the result with preproghrelin contained R28L-mutation, which prevented the cleavage of proghrelin to ghrelin (Fig. 6A). In this case, labeling of the proghrelin band was observed, but there was no ghrelin band. Labeled band could not be detected when the cells were transfected with catalytically inactive H338A-mutant of GOAT (Lane 5). As a further control, transfection of cDNA encoding another MBOAT (MBOAT1-a) failed to produce any labeled band (Lane 6). In a parallel immunoblot analysis, proghrelin was present in all 40%-CH_3CN fractions (Fig. 15B, Lanes 1-6), and octanoyl-ghrelin was only detected in Lane 2.

To verify that [^{3}H]octanoate was incorporated into proghrelin and ghrelin peptides without changing its length, I removed the labeled fatty acid from the peptides by methanolysis, and subjected the resulting methyl ester to thin-layer chromatography (TLC), which separated fatty acid methyl esters according to chain length (see **Experimental Procedures**). Scintillation counting of the TLC plate confirmed that the identity of ^{3}H-labeling attached to proghrelin and ghrelin was indeed eight-carbon octanoate (Fig. 15C). Consistent with the observation in mice and humans (Hosoda et al., 2003; Nishi et al., 2005), GOAT showed a specificity for medium-chain fatty acids such as octanoate. When transfected INS-1 cells were radiolabeled with [^{3}H]palmitate exactly as described for [^{3}H]octanoate in Fig. 15, I did not detect any incorporation of [^{3}H]palmitate into proghrelin or ghrelin (data not shown).

Relative Distribution of MBOAT mRNAs in Mouse Tissues

The expression patterns of preproghrelin, GOAT and other MBOAT mRNAs in various mouse tissues were compared using semi-quantitative RT-PCR (see **Experimental Procedures**, Fig. 16). As previously reported (Kojima et al., 1999), preproghrelin mRNA was expressed most highly in the mucosa of stomach followed by the intestine. There was very little expression in other tissues. Notably, the expression pattern of GOAT mRNA mirrored that of preproghrelin, i.e., highest in stomach, and detectable in the small intestine and colon. Among other organs, only testis and brain (including hypothalamus) showed small amounts of GOAT mRNA. None of the other MBOAT mRNAs matched the expression pattern of preproghrelin, and in fact, most were found in all of the tissues examined. Furthermore, the amount of GOAT mRNA in stomach appeared to be much lower than preproghrelin mRNA. Even after 35 cycles of PCR, the intensity of the amplified GOAT product was less than that observed with preproghrelin in only 30 cycles. This relative 200-fold difference in expression levels between the enzyme and the substrate was further confirmed in experiments using quantitative real-time PCR (data not shown).

Of note, the expression survey also revealed the distribution patterns of other MBOAT mRNAs besides GOAT. A large body of evidences suggest that Hedgehog and Wnt are critical signal components not only during animal development, but also in maintaining tissue homeostasis in adults (Jacob and Lum, 2007; Katoh, 2007). The finding of universal expression of HHAT and PORC mRNAs within all the tested mouse tissues seemed consistent with their roles in

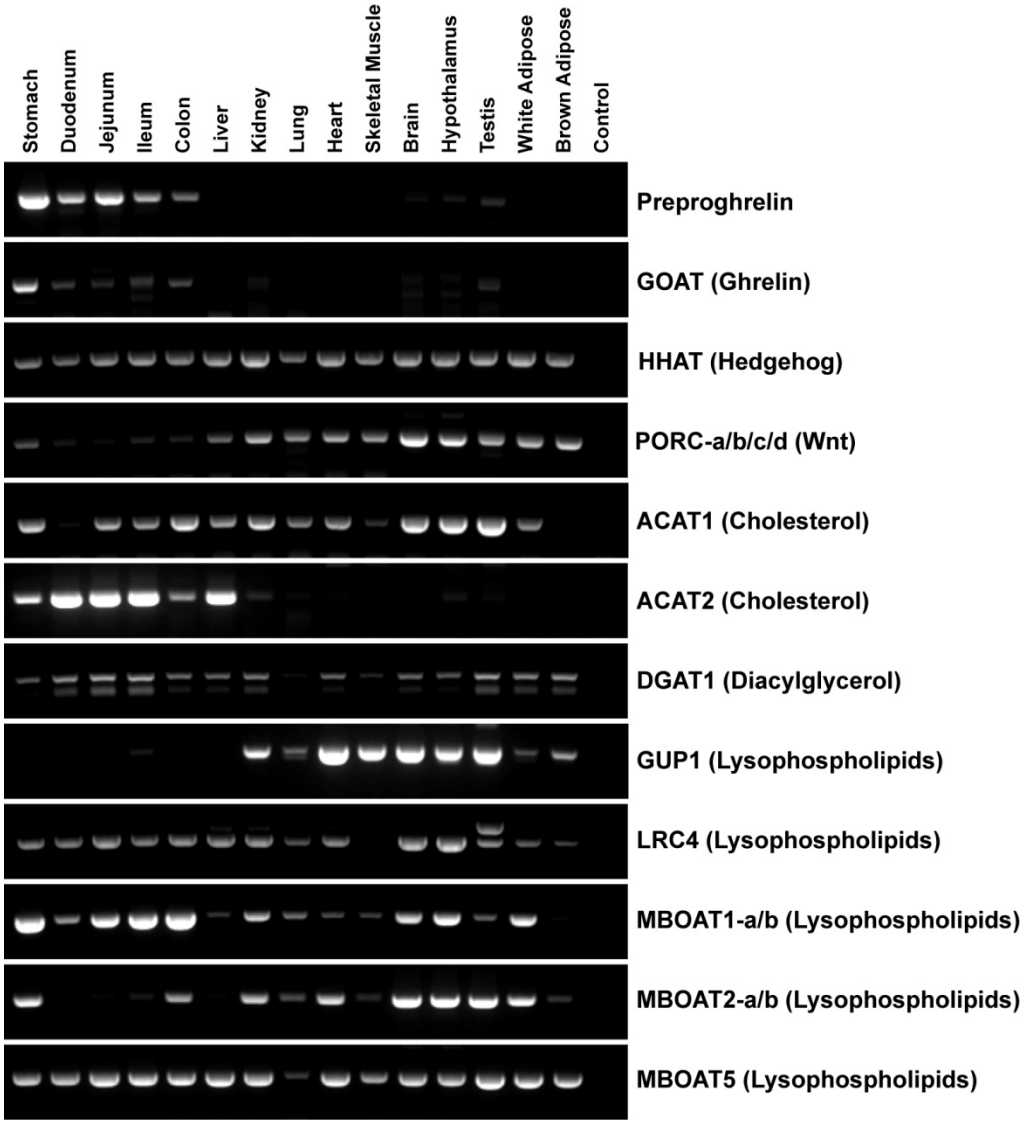

Figure 16. Tissue Distribution of Preproghrelin, GOAT and Other MBOATs
Relative expression level of indicated mRNA within a series of 15 difference mouse tissues was determined by semi-quantitative RT-PCR. The acyl-acceptor substrate for each MBOAT member is listed within the parentheses.

producing active signaling molecules. Recently, the substrates for orphan MBOAT family members have been characterized, including GUP1, LRC4, MBOAT1, MBOAT2 and MBOAT5 (Hishikawa et al., 2008; Jaquenoud et al., 2008; Lee et

al., 2008; Shindou and Shimizu, 2008). Intriguingly, those MBOAT members all function as lysophospholipid acyltransferases. LRC4 and MBOAT5 were detectable in most of the mouse tissues with only minor fluctuations in expression levels, whereas GUP1, MBOAT1 and MBOAT2 showed more individualized expression patterns. The absence of certain lysophospholipid acyltransferase in specific tissues, for instance, little or no GUP1 mRNA in gastrointestinal tract, could indicate that those tissues might possess a unique composition of phospholipids to accommodate their physiological functions.

Subcellular Localization of GOAT and Ghrelin

Studies with PC1/3-deficient mice have suggested that proteolytic processing and octanoylation of ghrelin could be two independent processes (Zhu et al., 2006). To gain insight into the sequential events in the production of octanoyl-ghrelin, subcellular localization of GOAT and ghrelin were individually determined by immunohistochemistry in transfected INS-1 cells (see **Experimental Procedures**).

As shown in Fig. 17, fluorescence signal of GOAT overlapped with cellular markers for ER and Golgi compartments, whereas no detectable co-localization of GOAT with secretory granules was observed, suggesting that the enzyme could reside in (or transport between) ER and Golgi. However, as a caveat, the experiments were carried out in transfected cells, and the presence of GOAT in ER and/or Golgi might be caused by overexpression of the recombinant protein. Due to the extremely low abundance of the enzyme, we were unable to visualize the endogenous protein with anti-GOAT antibody in mouse stomach (Dr. Tong-Jin

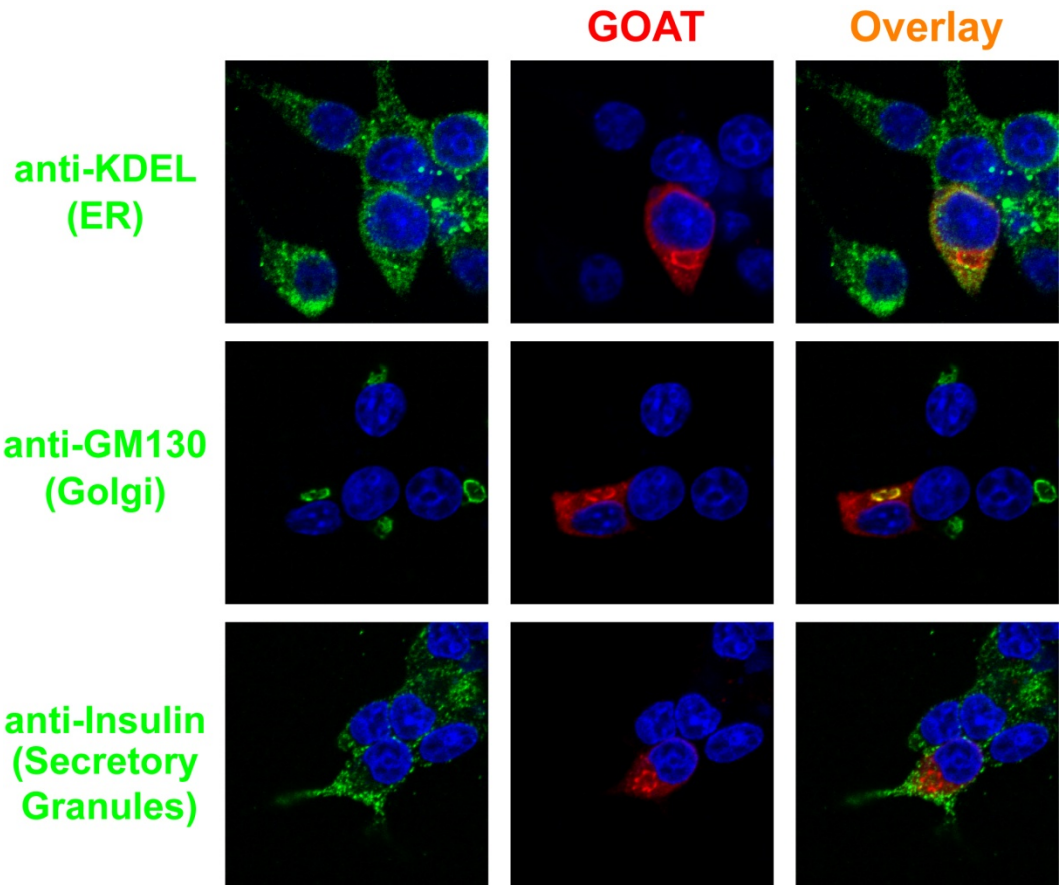

Figure 17. Subcellular Localization of GOAT in INS-1 Cells

INS-1 cells were set up in 24-well plate at a density of 1×10^5 per well on day 0, and on day 2, cells were transfected with 0.5 μg GOAT with C-terminal HA-tag. On day 4, cells were processed for immunohistochemistry of anti-HA antibody, in combination with anti-KDEL, anti-GM130 or anti-insulin to indicate the subcellular positions of ER, Golgi or secretory granules, respectively. Nucleus of individual cell was visualized by DAPI staining. Fluorescence signal of subcellular markers were shown in green, expression of GOAT in red, and nuclei in blue.

Zhao et al., unpublished data).

In contrast to its octanoylating enzyme, ghrelin showed exclusive localization in secretory granules, but not in ER or Golgi compartments (Fig. 18). The conclusion was further supported by immunoelectron microscopy (Fig. 19), in which gold particles were clustered in electron-dense vesicles corresponding to

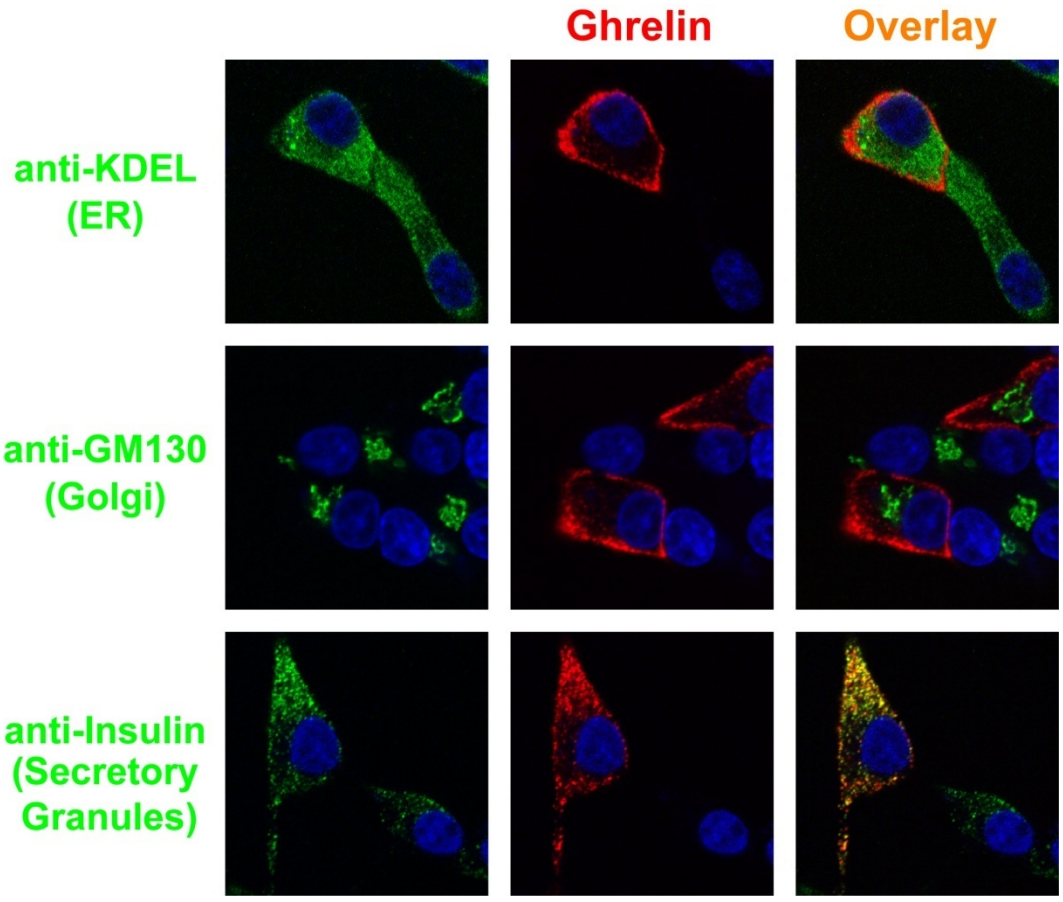

Figure 18. Subcellular Localization of Ghrelin in INS-1 Cells

INS-1 cells were set up in 24-well plate at a density of 1×10^5 per well on day 0, and on day 2, cells were transfected with 0.5 μg preproghrelin. On day 4, cells were processed for immunohistochemistry of anti-ghrelin antibody (94C), in combination with anti-KDEL, anti-GM130 or anti-insulin to indicate the subcellular positions of ER, Golgi or secretory granules, respectively. Nucleus of individual cell was visualized by DAPI staining. Fluorescence signal of subcellular markers were shown in green, expression of ghrelin in red, and nuclei in blue.

the classic structure of secretory granules. Since the amounts of ghrelin and proghrelin were comparable in transfected INS-1 cells (Fig. 5A), immunoreactive signal of ghrelin actually reflected the total population of the two peptides. The efficient partition of ghrelin (and also presumably proghrelin) into secretory granules suggested the protein transporting system in INS-1 cells sufficiently

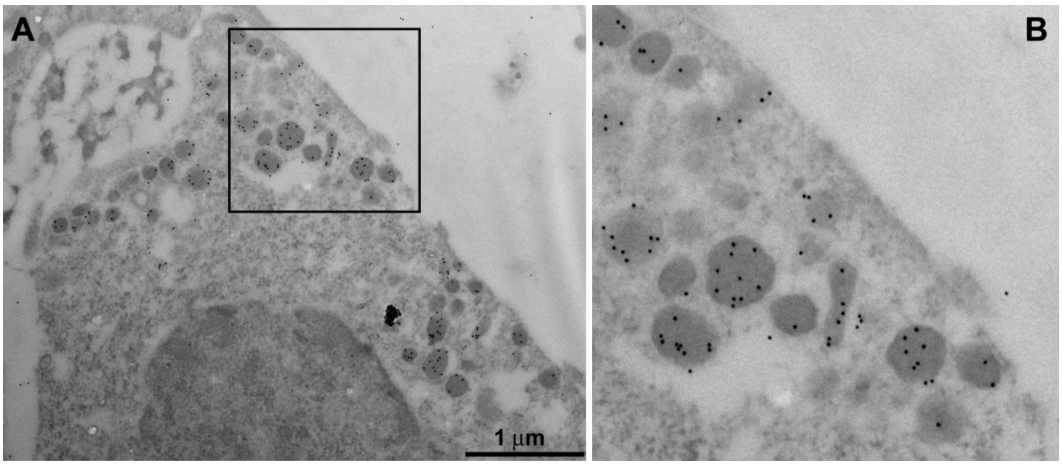

Figure 19. Immunoelectron Microscopy of Ghrelin in INS-1 Cells
(A) INS-1 cells were set up for experiments on day 0, and on day 2, cells were transfected with 6 μg preproghrelin. On day 5, cells were processed for immunoelectron microscopy as described in **Experimental Procedures**. Scale bar in the picture represents 1 μm.
(B) High power magnification of the indicated region in (A).

recognized the sorting signal on ghrelin. It will be interesting to determine whether the components involved in the sorting of insulin in INS-1 cells could also function to transport ghrelin from Golgi to secretory granules.

The identification of GOAT has pieced up a clear picture for the post-translational events in the production of octanoyl-ghrelin from its precursor (Fig. 20). After gene transcription and mRNA translation, preproghrelin gets inserted into ER lumen, where signal peptidase cleaves off the signal sequence to generate proghrelin. Since GOAT likely resides in ER and Golgi compartments, proghrelin will be octanoylated by the enzyme before its being further transported to Trans-Golgi Network (TGN), consistent with the findings that octanoylated proghrelin could be detected either in cultured cells (Fig. 15A) or in PC1/3-deficient mice (Zhu et al., 2006). The presumed donor for octanoyl group is octanoyl CoA, but how octanoyl CoA gets into ER lumen remained unclear.

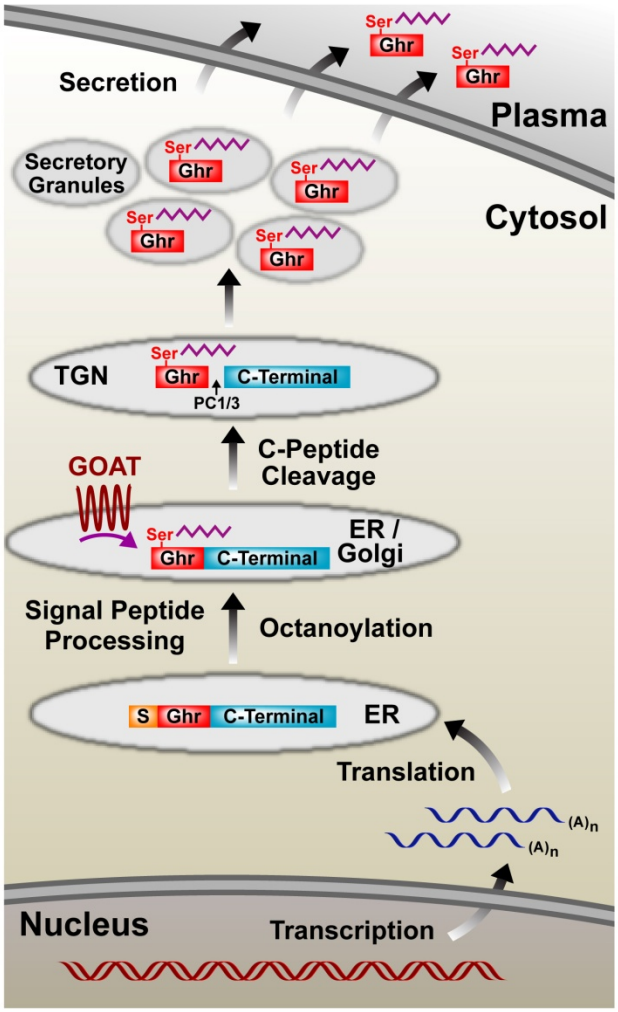

Figure 20. Post-Translational Processing of Ghrelin
The diagram shows sequential events in the production of octanoyl-ghrelin from its precursor. After transcription, ghrelin mRNA is translated into preproghrelin, which is co-translationally inserted into ER lumen. Signal sequence (S, in orange) of preproghrelin is cleaved off by signal peptidase, producing proghrelin with C-terminal peptide (C-Peptide, in blue) linked to ghrelin (Ghr, in red). In ER and Golgi compartments, GOAT octanoylates proghrelin by transferring octanoyl group (in purple) to Ser-3 residue. Upon its arrival in TGN, octanoylated proghrelin is cleaved by PC 1/3, resulting in mature form of octanoyl-ghrelin. Octanoyl-ghrelin finally gets sorted into secretory granules for secretion into plasma under physiological stimuli.

One possibility might be that GOAT itself couples the transfer of octanoyl CoA across ER membranes during the octanoylation reaction. Upon its arrival at TGN, C-terminal peptide of proghrelin is further processed by PC1/3 to generate mature octanoyl-ghrelin. Finally, ghrelin is sorted into secretory granules through an unknown mechanism, and gets ready to be secreted into plasma in response to physiological stimuli, e.g., food deprivation.

Recently, as the definitive proof of GOAT as the only octanoylating enzyme

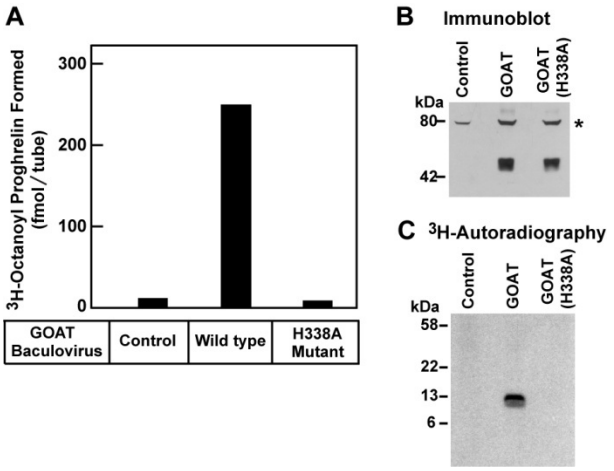

Figure 21. Establishment of *In Vitro* Octanoylation Assay

(A) GOAT activity in membranes from insect cells. Each 50-µl reaction mixture contained 50 µg membrane proteins from the cells infected with the indicated baculovirus, 5 µg proghrelin-His_8, 50 µM myristyl ether CoA, and 1 µM [^{3}H]octanoyl CoA (11 dpm/fmol). After incubation at 37°C for 5 min, the amount of [^{3}H]octanoyl transferred to proghrelin-His_8 was quantified by nickel chromatography and scintillation counting as described in **Experimental Procedures**. Each value represents the average of triplicate assays.

(B) Immunoblot analysis of GOAT expression in insect cells. Aliquots of the membranes used in (A) (75 µg of proteins) were subjected to immunoblot analysis with anti-His antibody. The asterisk (*) denotes an irrelevant cross-reacting band present in the membranes of insect cells.

(C) Autoradiography of [^{3}H]octanoyl proghrelin-His_8 formed *in vitro*. The reaction products from replicate assays in (A) were precipitated and subjected to autoradiography for 5 days as described in **Experimental Procedures**.

for ghrelin, octanoylation of the peptide was shown to be completely abolished in GOAT-deficient mice by gene-targeted deletion (Gutierrez et al., 2008) (Dr. Tong-Jin Zhao et al., unpublished data).

PART II: Biochemical Characterization of Ghrelin *O*-Acyltransferase

Establishment of Biochemical Assay for GOAT

All of the previously characterized members of MBOAT family transfer long-chain fatty acids to either lipids or proteins. GOAT is unique because it shows a strong preference to medium-chain fatty acids such as octanoate, and has

virtually been the only known enzyme that can attach medium-chain fatty acyl groups to a protein (or peptide). To better understand the enzymatic properties of this unusual acyltransferase, I established an *in vitro* assay for the octanoylation reaction. Such a biochemical assay has been important, since it will enable us to efficiently screen for small-molecule inhibitors for GOAT.

Sf9 insect cells were chosen as expression system for the recombinant enzyme, because large amounts of GOAT-containing membranes could be easily prepared. Insect cells were infected with baculovirus encoding mouse GOAT, and crude membrane fractions were isolated from disrupted cells by ultracentrifugation (see **Experimental Procedures**). Recombinant proghrelin with C-terminal His_8-tag was purified in *Escherichia coli*, which was used together with [^{3}H]octanoyl CoA as the two substrates for GOAT.

GOAT-containing membranes were incubated with proghrelin-His_8 in the presence of [^{3}H]octanoyl CoA, and the octanoylated proghrelin formed in the reaction was isolated by adherence to nickel-coated beads. As shown in Fig. 21A, membranes from uninfected cells transfer little amount of [^{3}H]octanoyl to proghrelin, but abundant transfer was seen with membranes from the cells expressing wild-type GOAT. Moreover, a comparable level of H338A-mutant GOAT, which mutation was catalytic inactive in transfected INS-1 cells (Fig. 14), failed to produce [^{3}H]octanoyl proghrelin (Fig. 21A and B), suggesting the octanoylating reaction reflected the activity of GOAT but not non-specific acyltransferases in crude membranes. To further confirm the direct attachment of [^{3}H]octanoyl to proghrelin, proteins were precipitated at the end of the reaction in Fig. 21A, and subjected to SDS-PAGE followed by autoradiography (see

Experimental Procedures). ^{3}H-radioactivity was detected only in the reaction containing wild-type GOAT, and incorporated into a band with an apparent molecular mass of 13 kDa, corresponding to proghrelin-His_8 (Fig. 21C).

In preliminary experiments, it was noted that GOAT activity was greatly enhanced when long-chain fatty acyl CoAs were included in the assays. The reaction was stimulated equally by myristoyl CoA (14 carbons), palmitoyl CoA (16 carbons), or oleoyl CoA(18 carbons) (Fig. 22A). The reaction was also stimulated by myristyl ether CoA, but not by free myristate. The addition of free CoA partially inhibited the reaction (Fig. 22B), possibly because it competed for the octanoyl CoA-binding site on GOAT.

The explanation for the apparent stimulation of GOAT activity by fatty acyl CoAs became apparent when I measured the amount of [^{3}H]octanoyl CoA that

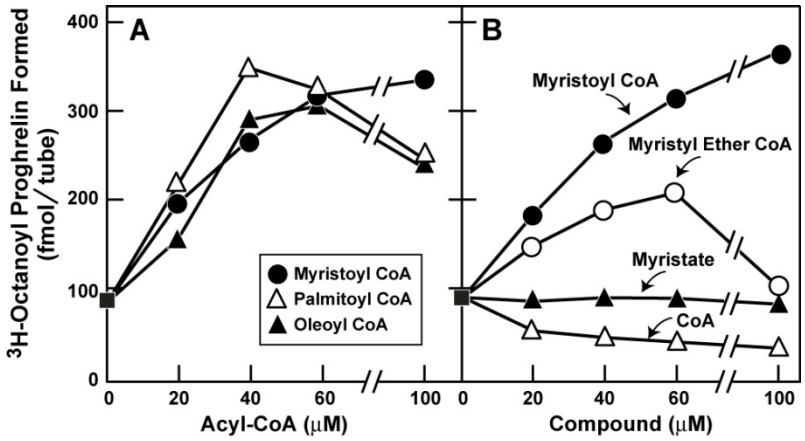

Figure 22. Stimulation of GOAT Activity by Long-Chain Acyl CoAs

(A and B) Membranes of insect cells expressing His_{10}-GOAT were prepared. Each 50-μl reaction contained 50 μg membrane proteins, 5 μg proghrelin-His_8, 1 μM [^{3}H]octanoyl CoA (11 dpm/fmol), and the indicated compounds. After incubation at 37°C for 5 min, the amount of [^{3}H]octanoyl transferred to proghrelin-His_8 was quantified by nickel chromatography and scintillation counting. Each value represents the average of duplicate assays.

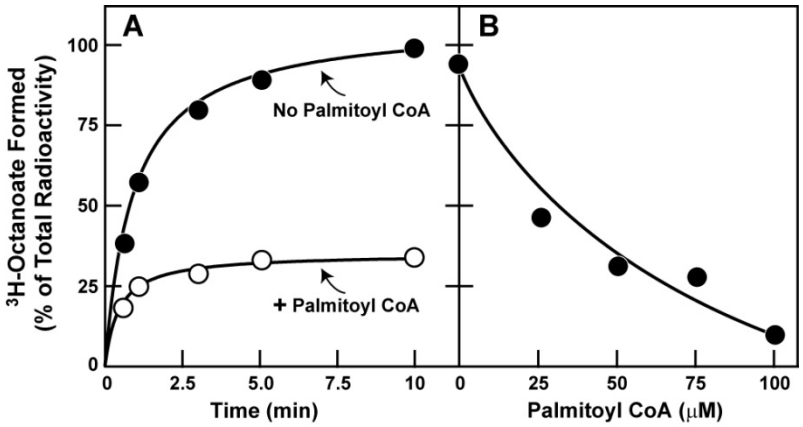

Figure 23. Long-Chain Acyl CoAs Inhibited Deacylation of [^{3}H]Octanoyl CoA

(A and B) Membranes of insect cells expressing His_{10}-GOAT were prepared. Each 50-μl reaction contained 50 μg membrane proteins, 5 μg proghrelin-His_8, 1 μM [^{3}H]octanoyl CoA (11 dpm/fmol), and 50 μM (A) or the indicated concentration of palmitoyl CoA (B). After incubation at 37°C for the indicated time (A) or 5 min (B), the amount of [^{3}H]octanoate formed in the reaction was quantified by TLC analysis as described in **Experimental Procedures**.

was cleaved to release [^{3}H]octanoate during the incubation. This cleavage was likely caused by deacylases that were present in GOAT-containing crude membranes, and no deacylation of [^{3}H]octanoyl CoA was observed when membranes were omitted from the reactions (data not shown). To quantify this deacylation process, I incubated [^{3}H]octanoyl CoA with GOAT-containing membranes, and used TLC to separate [^{3}H]octanoate from residual [^{3}H]octanoyl CoA (see **Experimental Procedures**). In the absence of palmitoyl CoA, more than 80% of [^{3}H]octanoyl CoA was converted to [^{3}H]octanoate within 5 min. In the presence of 50 μM palmitoyl CoA, less than 30% of [^{3}H]octanoyl CoA was de-acylated (Fig. 23A). At 5-min time point, the amount of [^{3}H]octanoate formed in the reactions declined as a function of the concentration of palmitoyl CoA (Fig. 23B by Dr. Tong-Jin Zhao).

The fact of long-chain acyl CoAs stimulating the octanoylation reaction

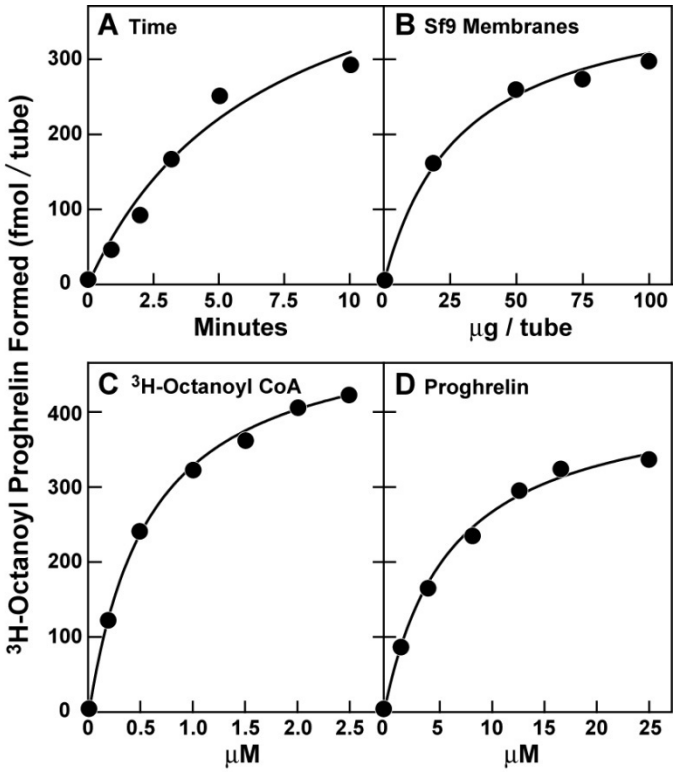

Figure 24. Characterization of GOAT Activity *In Vitro*
Membranes of insect cells expressing His_{10}-GOAT were prepared. Unless otherwise indicated, each 50-µl reaction contained 50 µg membrane proteins, 50 µM palmitoyl CoA, 5 µg proghrelin-His_8 (424,000 fmol), 1 µM [^{3}H]octanoyl CoA (11 dpm/fmol, 50,000 fmol). After incubation at 37°C for 5 min, the amount of [^{3}H]octanoyl transferred to proghrelin-His_8 was quantified by nickel chromatography and scintillation counting. Each value represents the average of duplicate assays.
(A) Time course. **(B)** Concentration curve of membrane proteins. **(C)** Concentration curve of [^{3}H]octanoyl CoA. **(D)** Concentration curve of proghrelin-His_8.

suggested that the recombinant GOAT showed a preference to medium-chain fatty acids in biochemical assays, consistent with the observations in animals and in culture cells. Currently, since no specific inhibitor for the deacylases in GOAT-containing membranes was readily available, all further assays were conducted in the presence of 50 µM palmitoyl CoA unless otherwise specified.

When assays were carried out at various pH values between 6.0 and 8.0, the highest rate of the reaction was at pH 7.0 in buffers containing either 50 mM Tris-Cl or 50 mM HEPES-Na. The following additions to the reaction had no significant effect on GOAT activity: 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM NaCl, 150 mM KCl, 400 mM sucrose, 1 mM EDTA, 1 mM DTT, and 1 mM

N-ethylmaleimide. Under the standard condition of *in vitro* assay, the formation of [^{3}H]octanoyl proghrelin was linear with time for 5 min (Fig. 24A). The reaction was not fully linear with the amount of GOAT-containing membranes (Fig. 24B), possibly caused by the presence of deacylases and other competing reactions. The reaction showed saturation kinetics with respect to the concentration of the two substrates [^{3}H]octanoyl CoA and proghrelin (Fig. 24C and D), and the apparent K_m values were 0.6 μM and 6 μM, respectively.

The kinetics of *in vitro* assay of GOAT were complex, owing in large part to the use of crude membranes as a source of the enzyme. Firstly, it was necessary to include long-chain acyl CoAs to prevent the deacylation of [^{3}H]octanoyl CoA. However, it could not be ruled out that the addition of long-chain acyl CoAs might result in competing reactions or directly affect the activity of GOAT.

Another question was related to the geometry of octanoylation reaction with respect to the cytosolic and luminal faces of membranes. Since proghrelin was octanoylated in the secretory pathway, the active site of GOAT must face the luminal side of membrane compartments. Thus, in order for GOAT-containing membranes to octanoylate proghrelin *in vitro*, at least a fraction of GOAT should be oriented with its active site facing the extraluminal side of membrane vesicles. I had attempted to increase this fraction of the enzyme by sonicating the vesicles, but did not observe an increase in GOAT activity (data not shown).

A critical question raised by the biochemical assay was the fate of the reaction product, namely, octanoylated proghrelin. In experiments not shown, all of [^{3}H]octanoyl proghrelin remained bound to GOAT-containing membranes. The observation that GOAT activity was subjected to end-product inhibition (see

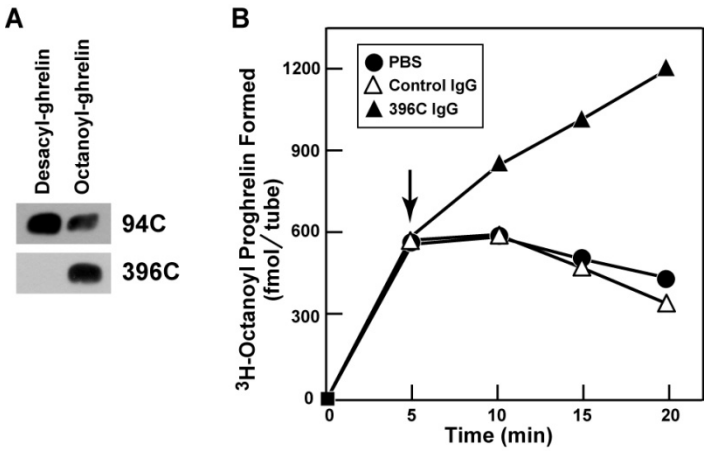

Figure 25. Neutralization of Octanoylated Proghrelin Extended the Reaction Over Time

(A) 396C antibody is specific to octanoyl-ghrelin. 10 ng of standard desacyl- and octanoyl-ghrelin were subjected to immunoblot analysis with purified IgG of 94C or 396C.

(B) 396C IgG extended the formation of [^{3}H]octanoyl proghrelin over time. Each 50-µl reaction contained 50 µg membrane proteins, 5 µg proghrelin-His_8, 50 µM palmitoyl CoA, and 1 µM [^{3}H]octanoyl CoA (11 dpm/fmol). After incubation at 37°C for 5 min, a final concentration of 0.1 mg/ml of control IgG or 396C IgG was directly added, and the reactions were further incubated at 37°C for indicated time. The amount of [^{3}H]octanoyl transferred to proghrelin-His_8 was quantified by nickel chromatography and scintillation counting.

below) seemed consistent with the idea that [^{3}H]octanoyl proghrelin could remain bound to the enzyme after its formation. Actually, it might account for the fact that octanoylation reaction was linear with time for only 5 min, when less than 1% of the two substrates, [^{3}H]octanoyl CoA and proghrelin, had been consumed. At that time point, a locally sufficient concentration of octanoylated proghrelin could have accumulated to inhibit GOAT for any further reaction. Supporting the idea, an addition of 396C IgG, which antibody was specific for octanoyl ghrelin (see **Experimental Procedures**), but not pre-immunized control IgG, efficiently extended the reaction over 5-min time point (Fig. 25). If such end-product inhibition also occurs in ghrelin-producing cells, then the cells must have some acceptor molecule(s) that removes octanoylated proghrelin from GOAT and allows the reaction to proceed catalytically.

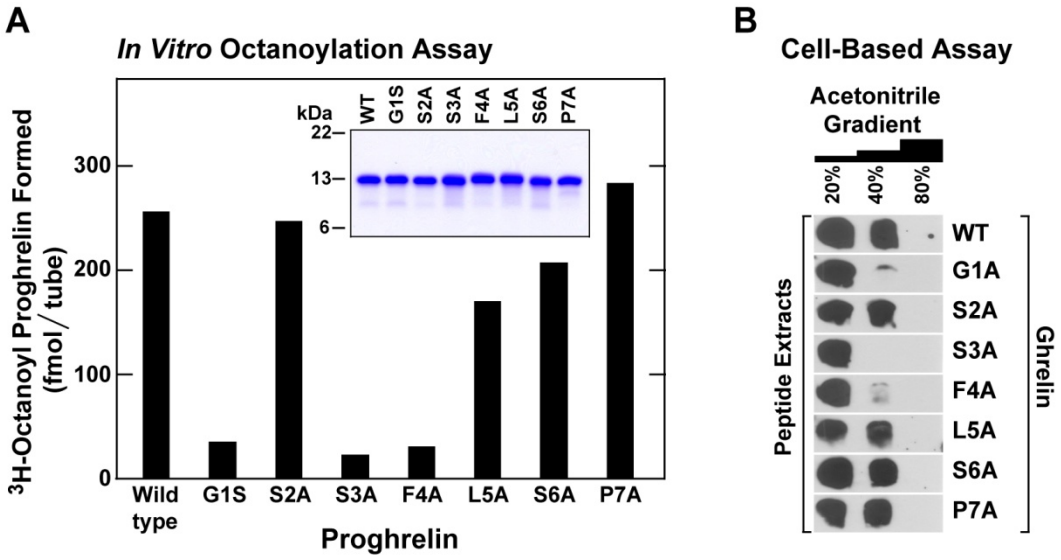

Figure 26. Identification of Amino Acids in Proghrelin Required for Octanoylation

(A) Octanoylation of mutant proghrelins *in vitro*. Each 50-µl reaction contained 50 µg membrane proteins, 5 µg of wild-type or indicated mutant versions of proghrelin-His_8, 50 µM palmitoyl CoA, and 1 µM [^{3}H]octanoyl CoA (11 dpm/fmol). After incubation at 37°C for 5 min, the amount of [^{3}H]octanoyl transferred to proghrelin-His_8 was quantified by nickel chromatography and scintillation counting. Each value represents the average of duplicate assays. (Inset) SDS-PAGE of purified wild-type and mutant versions of proghrelin-His_8. 5 µg of each recombinant protein was subjected to 16%Tricine SDS-PAGE and visualized by Coomassie Blue staining.

(B) Octanoylation of mutant ghrelins in INS-1 cells. INS-1 cells were set up for experiments on day 0, and on day 2, cells were transfected with 5 µg wild-type or indicated mutant versions of preproghrelin, together with 0.1 µg GOAT. On day 5, the peptides were extracted and subjected to reverse-phase chromatography.

GOAT Recognized N-Terminal Sequence of Ghrelin

To define the sequence requirement for substrate recognition by GOAT, recombinant proghrelin with a variety of Ala-mutations in the region of octanoylated serine were purified with help of Dr. Tong-Jin Zhao. As expected, replacement of Ser-3 with Ala nearly eliminated the formation of [^{3}H]octanoyl proghrelin, whereas replacement of the adjacent Ser-2 had no such effect. Replacement of N-terminal Gly-1 with Ser (or Ala, data not shown) markedly

reduced the octanoylation reaction, as did Phe-to-Ala substitution at residue 4 (Fig. 26A). Those results implicated Gly-1, Ser-3, and Phe-4 as components of the recognition sequence for GOAT. A much smaller effect was seen when Ala was substituted for Leu-5, and no inhibitory effect was observed when Ser-6 or Pro-7 was mutated (Fig. 26A).

The sequence requirement of *in vitro* octanoylation reaction was essentially identical to that observed in a cell-based assay. When transfected into INS-1 cells together with GOAT cDNA, wild-type ghrelin was efficiently octanoylated, but the formation of octanoyl-ghrelin was blocked when Gly-1, Ser-3, and Phe-4 were each replaced with Ala. Replacement of Ser-2, Leu-5, Ser-6, and Pro-7 had no significant effect (Fig. 26B by Dr. Tong-Jin Zhao). The identified recognition specificity was consistent with the fact that Gly-1, Ser-3, and Phe-4 of the peptide are absolutely conserved through all vertebrate ghrelins (Fig. 1B).

GOAT Octanoylated Ghrelin Pentapeptide

In addition to octanoylating proghrelin, GOAT also attached [^{3}H]octanoyl group to synthetic desacyl-ghrelin(1-28) *in vitro* as determined by autoradiography (Fig. 27A). When increasing amounts of desacyl-ghrelin(1-28) were added to the reaction in the presence of proghrelin-His_8, 50% inhibition of the formation of octanoylated proghrelin was achieved at a desacyl-ghrelin(1-28) concentration of 2 μM (Fig. 27A). A pentapeptide corresponding to the first five amino acids of ghrelin (GSSFL-COOH) was a weak inhibitor of the reaction (Fig. 27B). However, the inhibitory potency was increased markedly when C-terminus of the peptide was amidated (GSSFL-NH_2) with 50% inhibition achieved at 80

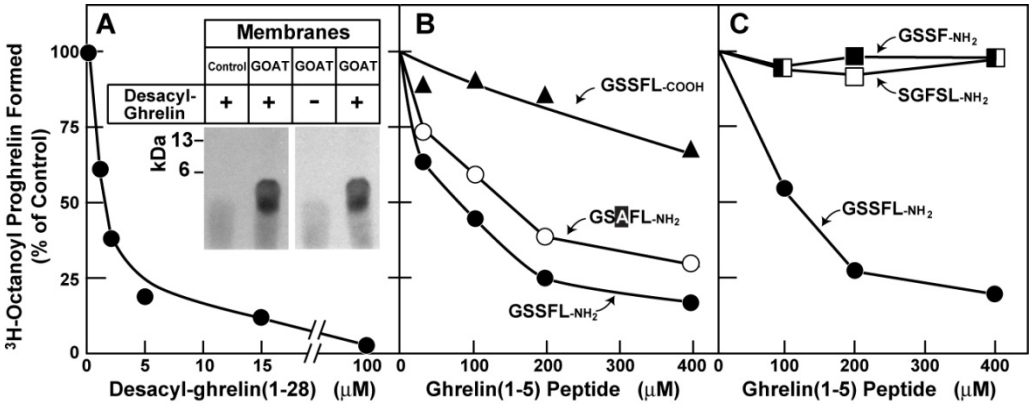

Figure 27. Desacyl-Ghrelin Peptides Competed for Octanoylation of Proghrelin

(A-C) Each 50-μl reaction contained 50 μg membrane proteins, 5 μg proghrelin-His_8, 50 μM palmitoyl CoA, 1 μM [^{3}H]octanoyl CoA (11 dpm/fmol) and the indicated concentration of peptides. After incubation at 37°C for 5 min, the amount of [^{3}H]octanoyl transferred to proghrelin-His_8 was quantified by nickel chromatography and scintillation counting. Each value represents the average of duplicate assays. "100% of control values", which represented the amount of [^{3}H]octanoyl proghrelin formed in the absence of peptides, were 278 (A), 247 (B), and 244 (C) fmol per tube, respectively. (A, inset) Autoradiography of [^{3}H]octanoyl ghrelin(1–28) formed in the *in vitro* assay. Each 50-μl reaction contained 50 μg membrane proteins from either uninfected insect cells (control) or cells infected with baculovirus encoding GOAT, 20 μM desacyl-ghrelin(1–28) as indicated, 50 μM palmitoyl CoA, and 1 μM [^{3}H]octanoyl CoA (11 dpm/fmol). After incubation at 37°C for 5 min, the samples were processed for autoradiography for 7 days as described in **Experimental Procedures**.

μM. This amidated peptide retained most of its inhibitory activity when Ser-3 was changed to Ala (GSAFL-NH_2) (Fig. 27B). In contrast, neither the amidated tetrapeptide (GSSF-NH_2) nor a scrambled amidated pentapeptide (SGFSL-NH_2) showed any inhibitory effect (Fig. 27C).

Next, I sought to determine whether GOAT could transfer [^{3}H]octanoyl to ghrelin pentapeptide (GSSFL-NH_2). For this purpose, GOAT-containing membranes were incubated with [^{3}H]octanoyl CoA and a saturating concentration of GSSFL-NH_2. At the end of the incubation, unlabeled authentic

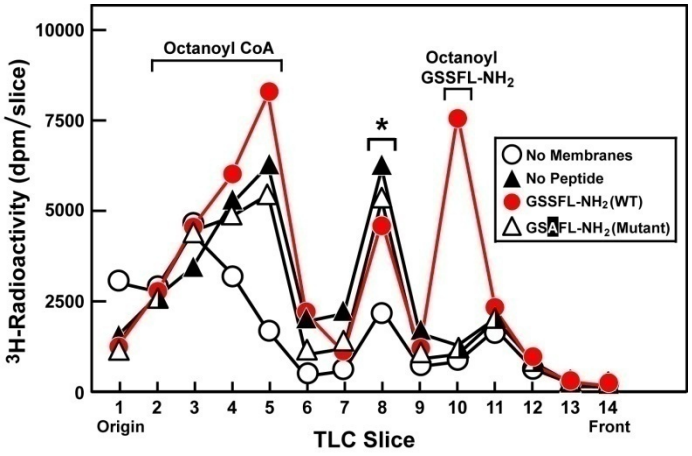

Figure 28. Octanoylation of Ghrelin Pentapeptide

Each 50-µl reaction contained 50 µg membrane proteins, 50 µM palmitoyl CoA, 1 µM [^{3}H]octanoyl CoA (11 dpm/fmol) and 500 µM indicated ghrelin pentapeptides. After incubation at 37°C for 10 min, the reaction mixtures from four tubes were pooled and subjected to TLC analysis as described in **Experimental Procedures**. The positions of the migration of residual [^{3}H]octanoyl CoA and authentic octanoyl-GSSFL-NH_2 are indicated. The identity of the middle peak of radioactivity, denoted by an asterisk (*), has not been characterized but is likely to represent membrane-dependent incorporation of [^{3}H]octanoyl group into an endogenous substrate.

octanoyl-GSSFL-NH_2 was added as a carrier. ^{3}H-labeled peptide was purified by reverse-phase chromatography, and then subjected to TLC analysis (see **Experimental Procedures**). Abundant formation of [^{3}H]octanoyl-GSSFL-NH_2 was seen when GOAT-expressing membranes were incubated with GSSFL-NH_2. There was no [^{3}H]octanoyl transfer when the membranes were omitted from the reaction or when the peptide contained Ala substitution for Ser-3 (GSAFL-NH_2) (Fig. 28). GSAFL-NH_2 pentapeptide inhibited GOAT with similar potency as GSSFL-NH_2 (Fig. 27B), even though it could not be octanoylated by the enzyme. Thus, desacyl ghrelin pentapeptides appeared to inhibit GOAT primarily by competing for the potential binding site, but not necessarily by serving as substrates for the octanoylation reaction.

Taken together, N-terminal five amino acids of ghrelin were both necessary and sufficient for GOAT-mediated octanoylation. Therefore, N-terminal segment

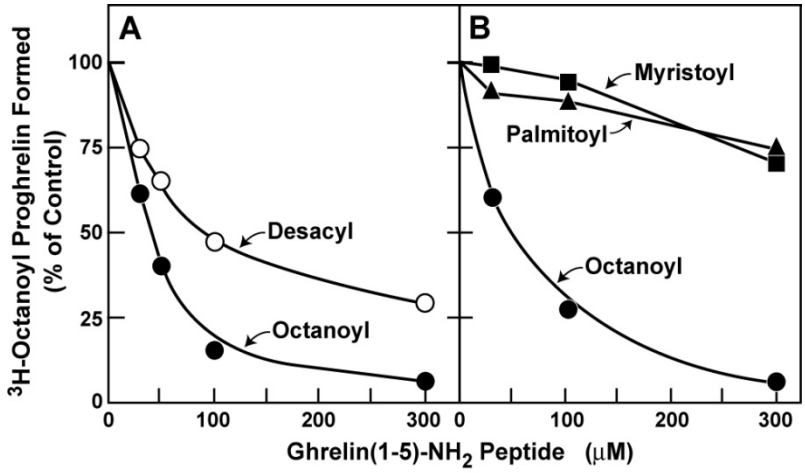

Figure 29. Inhibition of GOAT Activity by Octanoyl-Ghrelin(1–5)-NH_2 Peptide

(A and B) Each 50-µl reaction contained 50 µg membrane proteins, 5 µg proghrelin-His_8, 50 µM palmitoyl CoA, 1 µM [^{3}H]octanoyl CoA (11 dpm/fmol), and the indicated concentration of peptides in a final concentration of 3% (v/v) DMSO. After incubation at 37°C for 5 min, the amount of [^{3}H]octanoyl transferred to proghrelin-His_8 was quantified by nickel chromatography and scintillation counting. Each value represents the average of duplicate assays. "100% of control values", which represented the amount of [^{3}H]octanoyl proghrelin formed in the absence of peptides, were 205 (A) and 214 (B) fmol per tube, respectively.

of ghrelin serves two distinct yet convergent roles, i.e., a recognition site for octanoylation, and a core module for ghrelin binding to GHS-R (Bednarek et al., 2000), which could further emphasize its absolute conservation through evolution (Fig. 1B).

Potent Inhibition of GOAT by Octanoylated [Dap^3]-Ghrelin Peptides

Fig. 29A showed an experiment designed to test whether the addition of octanoyl group to ghrelin(1-5)-NH_2 (GSSFL-NH_2) would increase its potency as a GOAT inhibitor. When Ser-3 in GSSFL-NH_2 was octanoylated, its ability to inhibit GOAT increased relative to that of desacyl-ghrelin(1-5)-NH_2 peptide (50% inhibition at 45 µM vs. 80 µM, respectively). Interestingly, substitution of

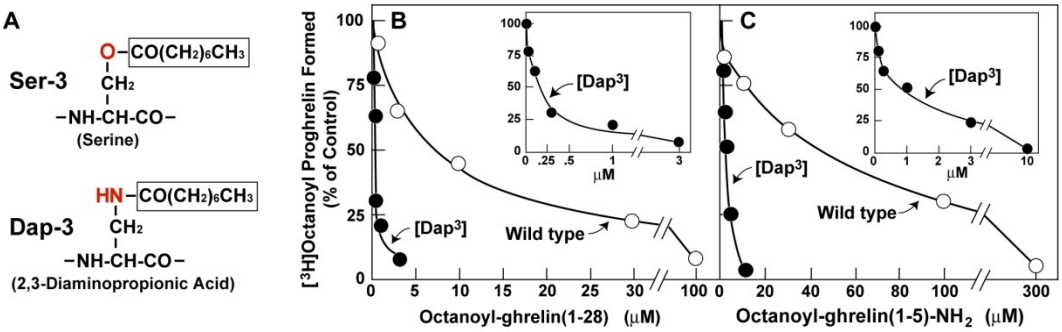

Figure 30. Inhibition of GOAT Activity by [Dap^3]Octanoyl-Ghrelin Peptides

(A) Diagram of octanoylated Dap residue. In contrast to octanoylated Ser, octanoyl group was attached to Dap through an amide-linkage.

(B and C) Each 50-μl reaction contained 50 μg membrane proteins, 5 μg proghrelin-His$_8$, 50 μM palmitoyl CoA, 1 μM [^{3}H]octanoyl CoA (11 dpm/fmol), and the indicated concentration of peptides in a final concentration of 3% (v/v) DMSO. After incubation at 37°C for 5 min, the amount of [^{3}H]octanoyl transferred to proghrelin-His$_8$ was quantified by nickel chromatography and scintillation counting. Each value represents the average of duplicate assays. (Insets) An expanded scale for the concentration curve of [Dap^3]octanoyl-ghrelin(1-28) (B) and [Dap^3]octanoyl-ghrelin(1-5)-NH$_2$ (C).

14-carbon myristoyl or 16-carbon palmitoyl for octanoyl group totally eliminated the inhibitory activity of ghrelin(1-5)-NH$_2$ (Fig. 29B by Dr. Tong-Jin Zhao).

Furthermore, I obtained a synthetic ghrelin(1-5)-NH$_2$ in which Ser-3 was replaced by (*S*)-2,3-diaminopropionic acid (Dap), and the distal amine of Dap-3 residue was octanoylated (Fig. 30A). With help of Dr. Tong-Jin Zhao, octanoylated [Dap^3]-ghrelin(1-5)-NH$_2$ was shown 45-fold more potent than octanoylated ghrelin(1-5)-NH$_2$ in blocking GOAT activity *in vitro* (50% inhibition at 1 μM vs. 45 μM, respectively) (Fig. 30C). A similar 35-fold increased potency was also observed when the octanoylated ghrelin(1-28) peptide contained Dap in place of Ser-3 (50% inhibition at 0.2 μM vs. 7 μM, respectively) (Fig. 30B). Considered together, the results demonstrated that GOAT could be subjected to end-production inhibition by either octanoylated ghrelin(1-28) or

ghrelin(1-5)-NH_2 pentapeptide. Moreover, the inhibitory potency was markedly enhanced when the octanoyl group was attached to ghrelin peptides through an amide linkage rather than its less stable ester counterpart, which insights may help guide the design of small-molecule inhibitors for GOAT.

CHAPTER III: DISCUSSION

Discovery of ghrelin has been a landmark not only in the research field of growth hormone, but also has unexpectedly revealed an important signaling molecule in appetite control. Mainly secreted from stomach, ghrelin has evolved as a peripheral hormone integrating the regulations of somatic growth and energy balance in human bodies (Kojima and Kangawa, 2005, 2006). As the unique feature of the peptide, octanoylation of Ser-3 residue of ghrelin has been the first example of protein *O*-acylation with a medium-chain fatty acid. Given the critical role of octanoylation in ghrelin endocrine functions, great interests have been aroused in the identity of the octanoylating enzyme.

By combining multidisciplinary approaches of bioinformatics, molecular biology and biochemistry, I have described here the identification of authentic ghrelin *O*-acyltransferase (GOAT), which had remained elusive for nearly a decade. Furthermore, I successfully established an *in vitro* assay for GOAT activity, with which several important biochemical features of the enzyme have been revealed. The discovery of long-missing GOAT has significantly advanced our knowledge on the production of octanoyl-ghrelin (Fig. 20). Moreover, the demonstration of end-product inhibition of the enzyme has opened up the possibility to design small-molecule inhibitors for GOAT, which may reduce appetite and diminish obesity in humans.

With those novel progresses, answers to several important questions have been immediately urged for a complete understanding of the biochemistry of GOAT and complexity of ghrelin physiology. Firstly, previous studies on protein

N-myristoylation and *S*-palmitoylation have identified three distinct classes of protein acyltransferases, i.e., *N*-myristoyl transferases, DHHC palmitoyl transferases and membrane-bound *O*-acyltransferases. *N*-myristoyl transferases show a strict specificity for 14-carbon myristate, whereas DHHC palmitoyl transferases have a preference for long-chain fatty acids. Except for GOAT, other MBOAT proteins all have a preference to long-chain or very-long-chain fatty acids, regardless of the substrates as lipids or proteins. Although the chemistry underlying ester-linkage acylation is basically identical, the question of how GOAT achieves its chain-length specificity in acylation reaction remains unclear. Detailed comparison of the catalytic domain of GOAT with those of other MBOATs, particularly HHAT and PORC, may help to reveal the residue(s) or region(s) determining the preference to medium-chain fatty acylation.

Another related question has been about the genomic structure of GOAT gene. All of the other MBOAT genes are consisted of 7 to 17 exons, with MBOAT domain in each protein separated into multiple exons. In contrast, GOAT gene is composed of only 3 exons, with the third exon encoding entire MBOAT domain. Furthermore, the exon composition of GOAT genes has been conserved from zebrafish to human. Although biological significance of such genomic structure is currently unknown, it could reflect the conserved function of GOAT during millions of years of evolution. Also, it is tempting to speculate whether certain exon(s) in common MBOAT domain has been missing in GOAT gene, which might result in the unique fatty-acid preference of the enzyme.

The general principle in processing of octanoyl-ghrelin from preproghrelin has become clear (Fig. 20), but one question has persisted regarding the source of

octanoate (or octanoyl CoA). In transfection experiments of INS-1 cells, I supplied exogenous octanoate in culture medium, which could be efficiently incorporated into ghrelin peptides (Fig. 15A). However, *de novo* synthesis of medium-chain fatty acids is known to be minimal in most mammalian cells. Therefore, where ghrelin-producing cells in the stomach get octanoate is unclear. One possibility is that dietary lipids could be a source, consistent with the finding of Nishi et al. (Nishi et al., 2005). Also, it can not be ruled out that a mechanism to synthesize medium-chain fatty acids might do exist in specialized cell types, which case has been shown in mammary glands (Knudsen and Grunnet, 1982). Moreover, the mechanism of how octanoyl CoA gets transported into ER lumen certainly needs further investigations.

Although the orexigenic effect of ghrelin has been well established, the neuro-humoral network in which ghrelin plays a critical function is largely unexplored. Given the evolutionary conservation of ghrelin, it has been puzzling in the field that deficiency of ghrelin and/or GHS-R in mice only resulted in very modest phenotypes (Sun et al., 2003; Wortley et al., 2004; Wortley et al., 2005; Zigman et al., 2005). Could it be caused by the different patterns of food intake between humans and rodents? Humans normally take meals with regular intervals (i.e., breakfast, lunch and dinner), whereas such a schedule is missing in rodents. It may explain that the oscillating rhythm of ghrelin levels in plasma has only been reported in humans (Liu et al., 2008). Otherwise, could it be reflecting the redundancy in neuro-humoral system, in which the loss of one regulator could be compensated by the rest parts? Since gene-targeted deletion of GOAT totally abolishes octanoyl-ghrelin (Gutierrez et al., 2008), the knockout mice could

surely serve as an important tool in addressing those questions. Also, a construction of drug-inducible GOAT-deficient mice will help to delineate the functions of octanoyl-ghrelin at different stages of development or under various physiological conditions.

Virtually, more than 70% of circulating ghrelin in human plasma is desacyl-ghrelin (Liu et al., 2008), which may represent an intermediate product during the decay of octanoyl-ghrelin. Although accumulating evidences have claimed specific roles of desacyl-ghrelin, the data from independent groups are controversial. For instance, conflicting results have been described regarding the effects of central (Asakawa et al., 2005; Toshinai et al., 2006) or peripheral (Asakawa et al., 2005; Inhoff et al., 2008) administration of desacyl-ghrelin on food intake. Also, studies with transgenic mice over-producing desacyl-ghrelin fail to show a consensus on the effect on plasma glucose level or food intake (Ariyasu et al., 2005; Asakawa et al., 2005; Iwakura et al., 2005). Moreover, desacyl-ghrelin does not activate GHS-R at physiological concentration (Kojima et al., 1999), and whether a specific and as-yet-unidentified receptor for the peptide exists need be addressed. Detailed characterization of GOAT-deficient mice will again become helpful to reconcile those contradictory data related to desacyl-ghrelin.

A major unanswered question in the field is why ghrelin has been the only known protein (or peptide) that needs to be octanoylated. This unique post-translational acylation is conserved in all vertebrates (Kojima and Kangawa, 2005). Besides for the binding of ghrelin to GHS-R, octanoylation of the peptide might hold other functions or enable layers of regulatory mechanism. For instance,

due to the hydrophobic feature of octanoyl group, the associations of desacyl- and octanoyl-ghrelin with lipoproteins show distinct profiles (De Vriese et al., 2007), which might control tissue distribution of the peptides. Also, a rapid deacylation of octanoyl-ghrelin in human plasma could serve as a regulatory role to stop the actions of the peptide. At least under certain physiological conditions, the octanoylation reaction itself may be regulated by the abundance of GOAT (Gonzalez et al., 2008), or by the availability of fatty acids (Liu et al., 2008; Nishi et al., 2005). Furthermore, it remains interesting in future to explore of whether ghrelin could exert any unappreciated function that links to its unique structure.

CHAPTER IV: EXPERIMENTAL PROCEDURES

Materials and General Methods

We obtained fatty acyl CoAs and myristyl ether CoA (tetradecanyl CoA) from Avanti Polar Lipids; detergents from Anatrace; [^{3}H-2,2',3,3']octanoyl CoA (60 Ci/mmol) and [^{3}H-2,2',3,3']octanoate (60 Ci/mmol) from American Radiolabeled Chemicals. Anti-Flag M2 Affinity Gel, bovine serum albumin (BSA, essentially fatty-acid free), solvents, and all other chemicals from Sigma unless otherwise specified.

Fatty acids were bound to BSA as sodium salts at a final concentration of 5 mM fatty acid and 10% (w/v) BSA in 0.15 M NaCl at pH 7.4 (Hannah et al., 2001). Delipidated fetal calf serum (FCS) was prepared by solvent extraction with *n*-butanol and isopropyl ether (Hannah et al., 2001). Standard methods of molecular biology were used unless otherwise specified.

Synthetic Peptides

All desacyl- and acylated versions of ghrelin(1-28) and ghrelin(1-5)-NH_2, including [Dap^3]octanoyl-ghrelin(1-28) and [Dap^3]octanoyl-ghrelin(1-5)-NH_2, were obtained from Peptide International (Louisville, KY) except for GSSFL-NH_2, GSSFL-COOH, GSAFL-NH_2, GSSF-NH_2, and SGFSL-NH_2, which were synthesized by Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center. Stock solutions for all peptides (1 or 10 mM) were made up in water except for palmitoyl-ghrelin(1-5)-NH_2, myristoyl-ghrelin(1-5)-NH_2, octanoyl-ghrelin(1-5)-NH_2, and

[Dap^3]octanoyl-ghrelin(1-5)-NH_2, which were made up in 100% DMSO.

Recombinant GST-Ghrelin and Proghrelin-His_8

DNA segments encoding mouse ghrelin(1-28) and proghrelin were cloned into pGEX-4T1 (GE Healthcare) to generate glutathione *S*-transferase (GST) fusion proteins. For GST-proghrelin construct, the thrombin cleavage site within the vector sequence (LVPRGS) between GST and proghrelin was changed to a Tobacco Etch Virus (TEV) protease site (ENLYFQG), and a His_8-tag was added to C-terminus of proghrelin. GST-ghrelin and GST-proghrelin-His_8 were expressed in *E. coli* and purified through glutathione-Sepharose beads (GE Healthcare). GST-proghrelin-His_8 was then cleaved by recombinant TEV protease to release proghrelin-His_8. TEV protease cleaved in such a way that the N-terminal sequence of proghrelin-His_8 was identical to that of authentic proghrelin. Proghrelin-His_8 was further purified by nickel-affinity chromatography (QIAGEN) and dialyzed with a 3,500 MWCO (Molecular Weight Cut-Off) dialysis membrane (Pierce) against 10 mM Tris-Cl (pH 8.5), 50 mM NaCl, 10% (v/v) glycerol, and 0.01% (w/v) CHAPS. The protein was concentrated with a 5,000 MWCO concentrator (Millipore) to a final concentration of 1 to 2 mg/ml, aliquoted and stored at -80^oC. Mutants of proghrelin-His_8 were generated by site-directed mutagenesis and purified as described above. The N-terminal sequences of all versions of recombinant proghrelin-His_8 were confirmed by Edman degradation.

Antibodies

Monoclonal anti-His antibody from GE Healthcare; monoclonal anti-KDEL (Grp78 / Grp94) antibody from Stressgen; monoclonal anti-GM130 antibody from BD Transduction Laboratories; monoclonal anti-insulin, monoclonal anti-Flag, and polyclonal anti-HA antibodies from Sigma; polyclonal anti-GST antibody from Invitrogen; polyclonal anti-MBP antibody from New England Biolabs. Donkey anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase from Jackson ImmunoResearch. Goat anti-mouse IgG conjugated to Alexa488 and Goat anti-rabbit IgG conjugated to Alexa568 from Molecular Probes.

For generation of polyclonal anti-ghrelin antibody, each rabbit was injected subcutaneously with 500 μg GST-ghrelin in incomplete Freund's adjuvant, followed by alternating booster injections of 250 μg GST-ghrelin or 250 μg proghrelin-His_8, both given subcutaneously in incomplete Freund's adjuvant at two week intervals. The resulting anti-ghrelin antiserum (94C) recognized proghrelin and ghrelin in both desacyl- and octanoyl-forms, but did not recognize C-terminal peptide derived from proghrelin cleavage.

Generation of polyclonal antibody against octanoyl-ghrelin followed the described strategy (Date et al., 2000). Briefly, synthetic octanoyl-ghrelin(1-11) with an extra Cys residue at C-terminus was conjugated with maleimide-activated keyhole limpet hemocyanin (Pierce). Each rabbit was injected with 500 μg antigenic conjugate followed by booster injections of 250 μg at two week intervals, all of which were given subcutaneously in incomplete Freund's adjuvant. The resulting anti-octanoyl-ghrelin antiserum (396C) recognized proghrelin and ghrelin in octanoyl- but not desacyl-forms. In experiments using IgG fractions, 94C and 396C antisera were purified by NAb Protein A/G Spin Kit (Pierce), and

buffer exchanged to Phosphate-Buffered Saline (PBS) using Zeba Desalt Spin Column (Pierce). Purified IgG was adjusted to a final concentration of 1 mg/ml, and stored in aliquots at -20^{o}C.

Bioinformatic Identification and Cloning of MBOATs

16 members of MBOAT family were identified bioinformatically in the mouse genome, using reported MBOAT sequences (Hofmann, 2000) for queries and PSI-BLAST searches (E-value cut-off 0.005, default parameters) (Altschul et al., 1997) against non-redundant protein sequence database.

Full-length cDNAs for all the MBOATs were cloned by reverse-transcription (RT)-PCR. Total RNA was isolated from the stomachs of C57BL/6J mice that had been either fasted for 16 hr or fed a chow diet *ad libitum*, and both sets of RNA were pooled together before RT-PCR. The cloned sequences with or without C-terminal Flag-tag (DYKDDDDK) or HA-tag (YPYDVPDYA) were inserted into pcDNA3 or pcDNA3.1 vectors driven by cytomegalovirus (CMV) promoter-enhancer. Primers for RT-PCR were designed according to the coding sequences available in National Center for Biotechnology Information (NCBI) database (Table 2). For each MBOAT without isoforms, 10 cDNA clones were sequenced in their entirety; for the three MBOATs with multiple isoforms (MBOAT1, MBOAT2, and Porcupine), 60 to 80 cDNA clones were sequenced.

Accession number (EU721729) for mouse GOAT has been deposited in GenBank Database. The chimpanzee ortholog (XP_519692) of GOAT was identified by BLASTP analysis of non-redundant protein database. Orthologs of GOAT in other species were found by clustering identified genomic sequences

with the SEALS command grouper (Walker and Koonin, 1997). In genomic sequence from several species, the annotation of exons did not contain the N-terminus of GOAT. In those cases, N-terminal amino acid sequence of mouse GOAT was used as a query to identify complete amino acid sequence of GOAT orthologs through TBLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST/). The reference numbers for the corresponding genomic DNA sequence were as follows: rat (NW_047474.1), human (NT_007995.14), bovine (NW_001494415.1), horse (NW_001799700.1), chicken (NW_001471685.1), and zebrafish (NW_001513480.1). All the sequence alignments were completed by ClustalW.

Mammalian Cell Culture and Transient Transfection

All cells were grown in monolayer at 37^{o}C in an atmosphere of 8.8% CO_2. Mouse AtT-20 cells (obtained from American Type Culture Collection) were cultured in Medium A (Dulbecco's modified Eagle's medium (4.5 g/L glucose) supplemented with 2 mM glutamine, 10% (v/v) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin). Rat INS-1 cells (Asfari et al., 1992) and mouse MIN-6 cells (Miyazaki et al., 1990) were obtained from Dr. Melanie Cobb (University of Texas Southwestern Medical Center). INS-1 cells were cultured in Medium B (RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin). MIN-6 cells were cultured in Medium C (Dulbecco's modified Eagle's medium (4.5 g/L glucose) supplemented with 10% FCS, 10 mM HEPES, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin).

For transient transfections, on day 0, AtT-20 cells were set up at 1×10^6 per 100-mm dish, INS-1 cells and MIN-6 cells were set up at 1.5×10^6 per 100-mm dish, unless otherwise specified. On day 2, cells were transfected with expression vectors using FuGENE HD Transfection Reagent (Roche) at a ratio of FuGENE HD to DNA of 3:1. On day 3 or 4, cells were subjected to various treatments. On day 4 or 5, cells were harvested for experiments. The total amount of transfected DNA in each experiment was constant and adjusted to 5 or 6 μg per 100-mm dish by addition of pcDNA3.1 mock vector.

N-Terminal Sequencing of Proghrelin and Its C-Terminal Peptide

INS-1 cells transfected with cDNA encoding preproghrelin with C-terminal Flag-tag were harvested on day 4 and washed once with PBS. Cells from 30 dishes were solubilized in PBS containing 0.1% (v/v) TritonX-100, 1 mM EDTA, and Protease Inhibitor Cocktail (Roche) at 4°C for 30 min. After centrifugation at 100,000*g* for 30 min at 4°C, a small aliquot of the supernatant (~1%) was subjected to 16% Tricine SDS-PAGE and immunoblotted with anti-Flag antibody. The remainder of the supernatant was treated with 200 μl of anti-Flag M2 Affinity Gel. After overnight incubation at 4°C, the bound proteins were eluted by boiling the beads at 95°C for 5 min in 25 mM Tris-Cl (pH 6.8) containing 1% SDS. After centrifugation at 20,000*g* for 5 min, an aliquot of the supernatant (~25%) was loaded onto 16% Tricine SDS-PAGE. Proteins were transferred to Immobilon-P^{SQ} PVDF membrane (Millipore) and stained with 0.1% (w/v) amido black in 5% (v/v) acetic acid. After destaining with 5% acetic acid, appropriate bands were excised from PVDF membrane and subjected to Edman degradation using Procise 494

Protein Sequencing System (Perkin-Elmer).

Peptide Extraction

Peptides were extracted from transfected cells using the protocol described by Kojima et al. (Kojima et al., 1999). After harvesting, cell pellets were boiled in 1 to 2 ml of H_2O for 10 min to inactivate proteases and then cooled down on ice, after which acetic acid and HCl were added to achieve final concentrations of 1M and 20 mM, respectively. The cell lysate was further disrupted by passage through a 22-gauge needle 10 times, followed by centrifugation at 20,000*g* for 10 min at 4^{o}C. The resulting supernatant was concentrated under vacuum to 20 - 30% of the original volume, subjected to 67% (v/v) acetone precipitation, and centrifuged at 20,000*g* for 10 min at 4^{o}C to remove the precipitates. The supernatant was finally evaporated under vacuum, and the residual materials were solubilized for immunoblot analysis, or for reverse-phase chromatography followed by immunoblot analysis as described below.

Immunoblot Analysis

For immunoblot analysis of proghrelin and ghrelin, the pellet containing extracted peptides was dissolved in SDS-PAGE loading buffer (0.1M Tris-Cl at pH 6.8, 5% (w/v) SDS, 0.1M DTT, and 5% (v/v) glycerol), subjected to 16% Tricine SDS-PAGE, and transferred to Immobilon-P PVDF membranes (Millipore). To prevent the diffusion of ghrelin peptide during blotting procedure, PVDF membrane was washed three times with PBS containing 0.05% (w/v) Tween-20, after which the membrane was fixed at room temperature for 10 to 15

min in 50 mM HEPES-NaOH (pH 7.4) containing 2.5% (v/v) glutaraldehyde. The membrane was then washed three times with PBS/Tween-20 and immunoblotted with 94C or 396C antisera.

To determine the expression level of MBOATs in transfected cells, cells from two dishes were harvested and washed once in PBS buffer. Cell pellets were disrupted in 1 ml of 10 mM HEPES-KOH (pH 7.6), 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose, 1 mM DTT, 2.5 μg/ml aprotinin, 20 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 10 μg/ml pepstatin-A by passage through a 22-gauge needle 30 times. After centrifugation at 3,000*g* for 5 min, the supernatant was centrifuged at 20,000*g* for 15 min. The membranes were dissolved in 200 μl SDS-PAGE loading buffer, and 20-μl aliquot of each sample was loaded onto 10% SDS-PAGE followed by immunoblot analysis with indicated antibodies.

Bound antibodies were visualized by chemiluminescence (Super Signal West Pico Developing Kit; Pierce) using either donkey anti-rabbit IgG or donkey anti-mouse IgG conjugated to horseradish peroxidase. All membranes were exposed to Phoenix Blue X-ray film for 5 s to 2 min at room temperature.

Reverse-Phase Chromatography of Desacyl- and Octanoyl-Ghrelin

Cells were treated with 50 μM octanoate-albumin overnight before harvesting. The extracted peptides were dissolved in 3 ml of 2% (v/v) CH_3CN / 0.1% (v/v) trifluoroacetic acid (TFA) and loaded onto a 360-mg Sep-Pak C18-cartridge (Waters). The cartridge was washed with 3 ml of 2% CH_3CN / 0.1% TFA, and eluted with a step-gradient of 6 ml of 20%, 40%, and 80% CH_3CN in 0.1% TFA.

The first 3 ml of each 6-ml elution were collected and evaporated under vacuum, and the residues were dissolved in 80 μl of SDS-PAGE loading buffer, and 20-μl aliquot of each sample was subjected immunoblot analysis.

Hydroxylamine Treatment

After evaporation of 40%-CH_3CN fraction of reverse-phase chromatography, the residual pellet was suspended in 0.4 ml of 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and Protease Inhibitors Cocktail (Roche). 0.2-ml aliquot was mixed with equal volume of either 2 M Tris-Cl (pH 8.0) or 2 M hydroxylamine (pH 8.0), and incubated at room temperature for 2 hr, after which the reactions were stopped by adding 0.5 ml of 1 M acetic acid. Each sample was further diluted in 10 ml of 2% CH_3CN / 0.1% TFA, and subjected to a second round of reverse-phase chromatography as described above.

[^{3}H]Octanoate Labeling and ^{3}H-Autoradiography

For metabolic labeling of [^{3}H]octanoate, two dishes of INS-1 cells were transfected with 5 μg preproghrelin (wild-type or mutant) and 0.2 μg either GOAT (wild-type or mutant) or MBOAT1-a as indicated. On day 3, cells were switched to Medium B containing 10% delipidated FCS instead of FCS. On day 4, cells were labeled in 8 ml Medium B containing no serum but supplemented with 1% (v/v) Insulin, Transferrin & Selenium Solution (ITS; Mediatech, Inc.), 0.1 mg/ml BSA, and 0.1 μM [^{3}H]octanoate (132 dpm/fmol). After incubation at 37^{o}C for 24 hr, peptides were extracted and fractionated by reverse-phase chromatography.

Each 40%-CH_3CN fraction was divided into two equal aliquots, each of which

was subjected to 16% Tricine SDS-PAGE. The separated proteins were transferred to PVDF membranes, after which one membrane was processed for immunoblot analysis, and the other for ^{3}H-autoradiography using Kodak Transcreen LE Intensifying Screen and Biomax MS Film at -80^{o}C for 5 days.

For determination of ^{3}H-labeled proghrelin-His$_{8}$ generated in the *in vitro* octanoylation assay, the reaction product was precipitated with 80% (v/v) of ice-cold acetone at -20^{o}C for 1 hr before centrifugation at 20,000*g* for 15 min at 4^{o}C. The resulting pellet was dissolved in 25 μl of SDS-PAGE loading buffer, loaded onto 16% Tricine SDS-PAGE, and subjected to ^{3}H-autoradiography as described above.

Thin-Layer Chromatography (TLC)

Identity of ^{3}H-labeled fatty acid linked to proghrelin and ghrelin in INS-1 cells was examined by fatty acid methyl ester (FAME) analysis. The pieces of PVDF membrane containing ^{3}H-labeled proghrelin and ghrelin in [^{3}H]octanoate-labeling experiment were cut out, pooled together, and treated with 0.5 ml of 0.1 M KOH in 100% methanol at room temperature for 2 hr to form FAME. After acidifying the sample with 0.5 ml of 1.0 M HCl, the aqueous phase was extracted twice with 0.1 ml hexane. 50-μl aliquot of the pooled organic phase was mixed with 50 μg of each FAME standard (methyl hexanoate, methyl octanoate, methyl decanoate, methyl dodecanoate, methyl myristate, and methyl palmitate) and spotted onto a C18 reverse-phase TLC plate (150 mm, 10×20 cm, Analtech). The TLC plate was developed in a solvent system of acetone/methanol/water (8:2:1, v/v/v), and FAME standards were revealed by iodine vapor counter-staining. The lane of TLC

was divided into strips numbered 1 to 14 from the origin to the front, with strips 6 to 11 containing individual FAME standards. The resin on each strip was then scraped off and subjected to liquid scintillation counting in 10 ml of counting cocktail (3a70B, Research Products International Corp.)..

For measuring the deacylation of [^{3}H]octanoyl CoA, each 50-µl reaction contained 50 mM HEPES-NaOH (pH 7.0), 50 µg membrane protein, 5 µg recombinant proghrelin-His_8, and 1 µM [^{3}H]octanoyl CoA (11 dpm/fmol) in the absence or presence of 50 µM palmitoyl CoA. After incubation at 37°C for 5 min, each sample was chilled on ice, and 200 µM palmitoyl CoA was added to stop further deacylation of [^{3}H]octanoyl CoA. 5-µl aliquot of each sample was spotted onto a Polygram SIL G plate (20×20 cm, Macherey-Nagel), and the plate was developed in a solvent system of chloroform/methanol/water (10:10:3, v/v/v). Each lane was cut into eight slices from the origin to the front, and the radioactivity in each slice was quantified by liquid scintillation counting. Standards of [^{3}H]octanoyl CoA and [^{3}H]octanoate were included in parallel lanes to indicate their positions of migration (R_f values of 0.0 and 0.9, respectively).

For identification of the transfer of [^{3}H]octanoyl from [^{3}H]octanoyl CoA to GSSFL-NH_2 pentapeptide, each 50-µl reaction contained 50 mM HEPES-NaOH (pH 7.0), 500 µM of the indicated pentapeptides, 50 µM palmitoyl CoA, and 1 µM [^{3}H]octanoyl CoA (11dpm/fmol) in the absence or presence of 50 µg membrane proteins. After incubation at 37°C for 10 min, the reaction was stopped by addition of 1 ml of 2% CH_3CN / 0.1% TFA containing 2.5 µg of unlabeled authentic octanoyl-ghrelin(1-5)-NH_2. Four assay tubes for each condition were pooled, loaded onto a 360-mg Sep-Pak C18-cartridge, and subjected to a

step-gradient of 20% (6 ml), 30% (12 ml), and 40% (3 ml) CH_3CN in 0.1% TFA. 20%- and 30%-fractions contained >90% of [^{3}H]octanoyl CoA and [^{3}H]octanoate but none of octanoyl-ghrelin(1-5)-NH_2, which was eluted in 40%-fraction. After evaporation of each 40%-fraction under vacuum, the residual pellet was dissolved in 20 µl of 40% CH_3CN / 0.1% TFA and spotted onto a Polygram SIL G plate, and the plate was developed in a solvent system of 1-butanol/acetic acid/water (4:1:1, v/v/v). After staining the plate with 0.2% (w/v) ninhydrin to denote the position of migration of octanoyl-ghrelin(1-5)-NH_2 (R_f value of 0.8), each lane of the plate was cut into 14 consecutive slices from the origin to the front, and the radioactivity in each slice was quantified by liquid scintillation counting.

MBOATs Expression in Mouse Tissues

Six-month old male C57BL/6J mice were fed a chow diet *ad libitum* prior to study. At the end of the dark phase, mice were anesthetized and exsanguinated. Various tissues were collected, snap-frozen in liquid nitrogen. The stomach, small intestine, and colon were flushed with cold PBS, after which the small intestine was divided into three equal lengths for duodenum (proximal), jejunum (medial), and ileum (distal). Each segment of the gastrointestinal tract was cut open, and the mucosa was carefully scraped off. Total RNA was prepared from mouse tissues using RNA STAT-60 kit (Tel-Test Inc., Texas, USA). Equal amounts of total RNA from four mice were pooled, treated with DNaseI (TURBO DNA-free, Ambion), and analyzed for mRNA expression of indicated genes using TITANIUM One-Step RT-PCR Kit (Clontech). Each reaction contained 1 µg of DNaseI-treated total RNA from different mouse tissues and primers shown in

(Table 3). The cycling parameters were set as 94^{o}C, 30 s; 60^{o}C, 30 s; and 68^{o}C, 30 s. Number of cycles was 30 (preproghrelin, ACAT1, ACAT2, GUP1, LRC4, MBOAT1, MBOAT2 and MBOAT5) or 35 (GOAT, DGAT1, HHAT and PORC). 20-µl aliquot of each 50-µl RT-PCR sample was loaded onto 1.5% agarose gel. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

Immunohistochemistry and Confocal Microscopy

INS-1 cells were set up on day 0 at a density of 1×10^{5} per well in 24-well plate with glass coverslip. On day 2, each well of cells were transfected with 0.5 µg preproghrelin or 0.5 µg GOAT with C-terminal HA-tag. On day 4, cells attached on coverslip were directly fixed in PBS containing 4% (w/v) paraformaldehyde at room temperature for 15 min. After permeabilization with PBST (PBS containing 0.1% (w/v) TritonX-100), cells were blocked in PBST containing 3% (w/v) BSA, and double-labeled with indicated primary antibodies: 1:200 of anti-KDEL, 1:500 of anti-GM130, 1:500 of anti-insulin, 1:500 of anti-HA, and 2 µg/ml purified 94C IgG. After washing three times in PBST, cells were then incubated with Goat anti-mouse IgG conjugated to Alexa488 and Goat anti-rabbit IgG conjugated to Alexa568, both at a dilution of 1:500 in PBST containing 3% BSA. After washing five times in PBST, each coverslip was dipped once in water, air dried and mounted onto slide with mounting media containing DAPI (VectaShield; Vector Laboratories). Imaging of the cells was carried out using Zeiss LSM510 META laser scanning confocal microscopy.

Immunoelectron Microscopy

INS-1 cells were set up for experiments on day 0, and on day 2, cells were transfected with 6 μg preproghrelin. On day 5, cells were fixed in PBS containing 4% paraformaldehyde and 0.1% (v/v) glutaraldehyde at room temperature for 1 hr. Immunoelectron microscopy was carried out by Molecular and Cellular Imaging Facility at the University of Texas Southwestern Medical Center. Briefly, after fixation, cells were enrobed in agarose, dehydrated through 85% ethanol, and embedded in LR White Resin. 70-nm thin sections were cut and placed on 200-mesh / Formvar-coated nickel grids for staining procedure with Leica IGL automated immunogold labeling machine. Primary antibody staining was in PBS containing 1% BSA and 5 μg/ml purified 94C IgG, and secondary antibody staining was in PBS containing 1:40 dilution of 12 nm gold-conjugated Goat anti-rabbit IgG (Jackson ImmunoResearch). Images were taken on a JEOL 1200EXII Transmission Electron Microscope operated at 120 kV, with a Sis Morada 11 Mega-Pixel CCD camera.

Insect Cell Culture and Baculoviral Infection

Mouse GOAT cDNA was inserted into pFastBac HT-A, which had N-terminal His_{10}-tag. Baculovirus was generated followed the manufacturer's instructions, and the titer of P3 virus was estimated to be 1×10^8 pfu/ml. Sf9 insect cells were cultured in Sf-900 II SFM, and set up on day 0 at a density of 5×10^5 cells per ml. On day 1, the cells were infected at a density of 1×10^6 cells per ml with baculovirus (multiplicity of infection ranging from 2 to 4). The cells were

harvested 24 to 48 hr post-infection, washed once with PBS, and stored at -80^{o}C.

In Vitro Ghrelin Octanoylation Assay

Each pellet of insect cells (1 liter of cell culture) was disrupted on ice with a Dounce homogenizer by 40 strokes on ice in 50 ml of 50 mM Tris-Cl (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μM bis (4-nitrophenyl) phosphate, 2.5 μg/ml aprotinin, 20 μg/ml PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin-A. After an initial centrifugation at 3,000*g* for 5 min at 4^{o}C, the supernatant was further centrifuged at 100,000*g* for 1 hr at 4^{o}C to obtain membranes, which were aliquoted and stored at -80^{o}C until the time of assay.

Prior to experiment, the membranes were suspended in 50 mM HEPES-NaOH (pH 7.0) and passed through a 22-gauge needle 10 times. After centrifugation at 1,000*g* for 1 min to remove aggregated materials, the supernatant (hereafter referred as membranes) was used for assays. Unless otherwise specified, the standard reaction contained 50 mM HEPES-NaOH (pH 7.0), 50 μg membrane protein, 5 μg recombinant proghrelin-His_8, 50 μM palmitoyl CoA, and 1 μM [^{3}H]octanoyl CoA (11 dpm/fmol) in a final volume of 50 μl. After incubation at 37^{o}C for 5 min, each reaction was stopped by adding 1 ml of Buffer A (50 mM Tris-Cl at pH 7.5, 150 mM NaCl, and 0.1% (w/v) SDS), after which each sample was loaded onto a 0.2-ml nickel-affinity column. The columns were washed at room temperature with 1 ml of Buffer A followed by 3 ml of Buffer A containing 25 mM imidazole. His-tagged proghrelin was then eluted with 1 ml of Buffer A containing 250 mM imidazole. Radioactivity present in the 250 mM-imidazole eluate was quantified by liquid scintillation counting. Blank values were

determined in parallel 50-µl reactions containing all components except proghrelin-His_8. These values ranged from 6 to 10 fmol per tube, and were subtracted from all the data points of experiments.

CHAPTER V: REFERENCES

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Table 1. Cell Lines Screened for Proteolytic Processing of Preproghrelin

Name	Species	Type	Name	Species	Type
3686	Human	Epidermal Carcinoma	CRL-7869	Human	n/a
7315a	Rat	Pituitary Tumor	CHO-7	Hamster	Ovary
A-204	Human	Rhabdomyosarcoma	Du-145	Human	Prostate Carcinoma
A-375	Human	Melanoma	F-3	n/a	n/a
A-431	Human	Epidermal Carcinoma	FEC	Cat	Embryo
A-498	Human	Kidney Carcinoma	H-1792	Human	Lung Adenocarcinoma
A-549	Human	Lung Carcinoma	H-2052	Human	Mesothelioma
A-1163	n/a	n/a	HEK-293	Human	Kidney
A-1165	Human	Pancreatic Carcinoma	HeLa	Human	Cervical Adenocarcinoma
A-2183	n/a	n/a	HEP-2	n/a	n/a
A-3733	n/a	n/a	HepG-2	Human	Hepatocellular Carcinoma
A-3827	Human	Astrocytoma	HT-29	Human	Colon Adenocarcinoma
AtT-20	Mouse	Pituitary Tumor	HTB-14	Human	Glioblastoma
BPA-31	Mouse	n/a	HTB-16	Human	Glioblastoma
C-46	Mouse	Neuroblastoma	HTB-17	Human	Glioblastoma
CCL-17	Human	Oral Epideimoid	HTB-22	Human	Breast Adenocarcinoma
CCL-23	Human	Epidermal Carcinoma	HTB-30	Human	Breast Adenocarcinoma
CCL-121	Human	Fibrosarcoma	HTB-103	Human	Gastric Carcinoma
CRL-1435	Human	Prostate Carcinoma	HTB-105	Human	Embryonal Carcinoma
CRL-1739	Human	Gastric Adenocarcinoma	INS-1	Rat	Insulinoma
CRL-1740	Human	Prostate Carcinoma	MIN-6	Mouse	Insulinoma
CRL-1803	Human	Thyroid Medulla Carcinoma	NRK-SA6	Rat	n/a
CRL-2266	Human	Neuroblastoma	PC-12	Rat	Pheochromocytoma
CRL-5815	Human	Lung Carcinoid	SV-80	n/a	n/a
CRL-5822	Human	Gastric Carcinoma	T11G9	n/a	n/a
CRL-7478	Human	n/a	Y-1	Mouse	Adrenal Tumor

Table 2. Sequences of Nested Primers for 5'-RACE of MBOAT4

Set 1	RT-primer	GGAGATAAGCAATGTAAAG
	Outer-primer	CCCAACAGAGGGGTGGAGCCATGGAAAC
	Inner-primer	CTCTCCCCTCGCAGTGTTTACTGAAAAG
Set 2	RT-primer	CTAAGTGGTGCCCTTGTG
	Outer-primer	CACAACTGGGCTCTCCGTAGCCTTTTTATAG
	Inner-primer	GGATGTAGAAAGGCCTGCCGGACAAAATG
Set 3	RT-primer	GAAGCGCAAAGGGGAATG
	Outer-primer	GAATGCAGCCCCTTGATAAAATGATAAAGGATG
	Inner-primer	GAGCTGGAGCCAATCCATCCTGAATGTG
Set 4	RT-primer	GAAGATGAGCAGAGAGTAG
	Outer-primer	GCCAGGACACCTCCTCCAGCCAGGAGAAAG
	Inner-primer	CCTGGCCCGGGTGGAAAAGGTGTCCAAG
Set 5	RT-primer	CTCACCCAGGTAGTATTC
	Outer-primer	CGGTGTAGTGAAGACCCAGATGGCACAG
	Inner-primer	GGGTCTGCCAGCCCATCTGAAAGAAGAAG

Table 3. Primer Sequences for cDNA Cloning of Mouse MBOATs

Gene	Sequence of forward and reverse primers (5'–3')	Accession Number
ACAT1	ATGTCACTAAGAAACCGGC CTAAAACACGTACCGACAAG	NM 009230
ACAT2	ATGCAGCCAAAGGTGCCCC CTAGGGATGGCAGGACCAAGAG	NM_146064
DGAT1	ATGGGCGACCGCGGAGGCGC TCAGACCCCCACTGGGGCATCG	NM_010046
GOAT	ATGGATTGGCTCCAGCTCTTTTTTCTGCATCCTTTATC TCAGTTACGTTTGTCTTTTCTCTCCGCTAACAG	XM_001476434
GUP1	ATGGGCATCAAGACAGCACTGC CTACTCCAGCTTCTCTCTGTCC	NM_029095
HHAT	ATGCTGCCCGGGTGGGAACTG TTAAAGAACTGTGTATGTCTGAG	NM_144881
LRC4	ATGACACCCGAAGAATGGAC TCACTCTTCCCGGAGCTTTTCC	NM_029934
MBOAT1-a & b*	ATGGCAGCACGGCCGCCCGC TCAGTCTGCCTTCTTCTTTACAG	NM_153546
MBOAT2-a	ATGGCCACCACCAGCACCAC TCACTGTGTTAGTGACGAGTGTC	NM_001083341
MBOAT2-b	Same as MBOAT2-a	NM_026037
MBOAT5	ATGGCGTCTACAGCGGACGG TCATTCCCTCTTTTTTAACTTTTCTTTTCTTGG	NM_145130
PORC-a	ATGGCCACCTTCAGCCGCCAG TCAGCCTATGAGACGGTAGAAGATCC	NM_016913
PORC-b	Same as PORC-a	NM_145908
PORC-c	Same as PORC-a	NM_145907
PORC-d	Same as PORC-a	NM_023638

* We identified two isoforms of MBOAT1, designated MBOAT1-a and MBOAT1-b, which differ from each other by a 32 amino-acid in-frame deletion in MBOAT1-b (Y108 through G139).

Table 4. Primer Sequences for Tissue Distribution Survey

Gene	Sequence of forward and reverse primers (5'–3')
Preproghrelin	ATGCTGTCTTCAGGCACCATCTGCAGTTTG
	TTACTTGTCAGCTGGCGCCTCTTTGACCTCTTC
GOAT	GGAAGTCCATAGGCTGACCTTCTTCTTTCAGATG
	GAACGCAAGCCTGAAACCTCCGGAAGGAAC
HHAT	GCACAGACCCTGGATTGTCATGGTCTATG
	GAGGCACCTTGCGCAGATGGAGTAGGTTG
PORC-a/b/c/d	GGCGTCTTCCTCTCCGTCACCATCCTCATC
	CCAGGCTCCGGGCCACCTTCTTC
ACAT1	GAGCCAAAGATCTGAGAGCACCTCCAGAAC
	GCCATGGACAAGGGAATAGATCAATGGGTG
ACAT2	CACTGATCGATGAGCTAATGGAGGTGCAAC
	ACAGCCACAGGGTCTGGTAGGGCACTAG
DGAT1	GGTGTGTGGTGATGCTGATCCTGAGTAATGC
	CAAACACGGAACCCACTGGAGTGATAGACTC
GUP1	CACGTGCTGTTTGCCAAACTCTGCACGATG
	CGAAGCTGGTGCACCGCAGAACTGTGAAG
LRC4	GCCCACTCCCACGCCCTTCACCAATG
	GGAGGACAGCAGGAAGAGCAGGCCAAAG
MBOAT1-a/b	GTCACTCAGAAGATCACGACGTTGGCTTTC
	CGGGAAAGGACTTGGAGAGCGTCAAAAACAAC
MBOAT2-a/b	GGCAGGCCCACTGTGCTCCTACAAAG
	GGCAGCCAAGAGAGAGACGTACAGATAG
MBOAT5	CGTGCTTCAGTTCCTCATCCTGCGACTCATG
	CCCGCACCAGCTTCATGTAGTGGTTCATTG